that pregnandiol excretion will help to remove doubt, for while the occurrence of pregnandiol excretion may be merely the normal prelude to a fresh menstrual bleeding, its persistence at a subsequent estimation a fortnight or more later renders this very unlikely and a diagnosis of pregnancy almost certain.

Summary

Pregnandiol is a product of corpus luteum and placental metabolism. Its excretion in the urine is relatively easily measured in any biochemical laboratory. An attempt has been made to assess the diagnostic value of pregnandiol excretion in abnormal pregnancy. Its complete absence is nearly always evidence of serious abnormality, suggesting in early pregnancy the imminence of abortion and in late pregnancy the death of the foetus. The significance of a low pregnandiol excretion still remains uncertain. A normal pregnandiol excretion is not evidence that abortion will not occur or that the foetus is still alive. Both chronic nephritis and toxæmia of pregnancy may interfere with pregnandiol excretion. Deviations from the normal excretion in these conditions do not necessarily mean that the gestation is abnormal. Pregnanndiol excretion seems assured of a place as a diagnostic aid in obstetric disorders.

REFERENCES

- Browne, J. S. L., Henry, J. S., and Venning, E. H. (1939). *Amer. J. Obstet. Gynec.*, **38**, 927.
 Coppe, C. L. (1940a). *Lancet*, **2**, 158.
 — (1940b). *Clin. Sci.*, in the press.
 Hain, A. M. (1940). *J. Endocrinol.*, **2**, 104.
 — and Robertson, E. M. (1939). *Lancet*, **1**, 1324.
 Venning, E. H. (1937). *J. biol. Chem.*, **119**, 473.
 — (1938). *Ibid.*, **126**, 595.
 — and Browne, J. S. L. (1937). *Endocrinology*, **21**, 711.
 Weil, P. G. (1938). *Science*, **87**, 72.
 Wilson, R. B., and Randall, L. M. (1938). *Proc. Mayo Clin.*, **13**, 813.
 — (1939). *Ibid.*, **14**, 8.

EFFECT OF REPEATED INTRAVENOUS INJECTIONS OF BILIRUBIN ON ERYTHROPOIESIS IN ANAEMIC DOGS

BY

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It is well known from animal experiments that regeneration after bleeding occurs more rapidly if a solution of the haemoglobin removed is reinjected into the anaemic animal (McMaster and Haessler, 1921; Miller and Rhoads, 1934). To explain this observation it has sometimes been assumed that as the injected haemoglobin is broken down some product or products are retained and re-utilized in the formation of new haemoglobin. The experiments of Hawkins *et al.* (1931, 1939) have shown, however, that this does not happen in the case of the pigment portion; since under experimental conditions in dogs with bile-renal fistulae 90 to 100 per cent. of pigment injected as haemoglobin is excreted in the urine, although at the same time an amount of new haemoglobin equivalent to 90 to 100 per cent. of that injected appears in the circulation. Other observations recorded in the literature and reviewed below indicate that injections of bilirubin do have an effect on erythropoiesis. If this effect is not due to an actual utilization of bilirubin in the formation of new haemoglobin it must be due to some other and at present unknown action of bilirubin on erythropoiesis.

Previous Observations

Patek and Minot (1934) reported the results of treatment with bile pigment in nine cases of chronic hypochromic anaemia. A concentrated almost pure preparation of beef bile pigments was given by mouth in doses

varying from 1.2 to 2.5 grammes daily. This preparation given alone caused no reticulocyte response, but there was an average increase of approximately 7 per cent. in the amount of haemoglobin in about ten days. After a reticulocyte response had occurred as a result of the administration of a suboptimal dose of iron the addition of the bile-pigment preparation caused a second response. In one patient who had been treated with iron for fourteen months without reaching a haemoglobin level higher than 73 per cent. this rose to 85 per cent. in sixteen days when bile pigment was given in addition to iron. These observations seem to indicate that bile pigment given by mouth can in some manner facilitate either the absorption or the utilization of iron. It is open to question, however, whether they also show, as their authors claim, that bile pigment can be absorbed from the gastro-intestinal tract and utilized in the building of haemoglobin.

There are a number of articles in German describing experiments on the effects on erythropoiesis of bile and blood pigments given by mouth and by injection. Verzar (1929), for instance, claimed that various blood pigments, including haemoglobin, bilirubin, and porphyrins, as well as various organ extracts, were erythropoietic. He found that single doses of bilirubin given by mouth or by injection to rabbits caused a transient erythrocytosis in some animals—usually animals with a red cell count in the lower part of the normal range; an erythropenia was, however, produced in animals with a higher count. Regeneration after bleeding was said to occur more rapidly in animals given bilirubin intravenously or by mouth, but the diagrams illustrating this are hardly convincing and the results were apparently not consistent. Fellingner (1932) also found that a single parenteral injection of bilirubin caused an increase in the red cell count of normal rats in about half the animals so treated; and this occurred especially in young animals and in animals with lower red cell counts than the remainder. Continued administration led to a more prolonged increase, which ceased when the medication was discontinued. Regeneration after bleeding was said to be more rapid in animals treated with bilirubin than in animals not so treated; but in this instance again the protocols are not entirely convincing.

Zih (1939) found that the effect of intravenous injections of bilirubin in normal men depended on the dose given. A single dose of 10 to 30 mg. produced an erythrocytosis of short duration, but 150 to 300 mg. produced an erythropenia. Biliverdin gave a similar result, while haematoporphyrin had very little effect. A solution of 15 grammes or more of commercial haemoglobin taken by mouth also produced an erythrocytosis.

The effects of bilirubin on haematopoiesis are therefore, according to these authors, somewhat complicated. There is some evidence that bilirubin in small doses produces erythrocytosis, but that in larger doses it produces erythropenia. Erythrocytosis is most often seen in animals which have initially a red cell count in the lower part of the range of normal, and erythropenia may be seen in those with a higher red cell count. These authors have speculated as to the significance of the effects observed and have even ascribed to bilirubin the function of an erythropoietic hormone concerned in the maintenance of normal blood values. Some of their experiments are, however, open to criticism in that they were performed on small animals on which accurate blood counts are difficult, and the changes observed, which were slight, were sometimes not consistent. The experiments were of short duration, and too often red cell counts alone are recorded.

The object of the experiment reported here was to discover the effect of intravenous injections of bilirubin, given

over a considerable period, on the rate of production of haemoglobin, the size of the red cells, and the reticulocyte count of anaemic dogs. The dogs were made anaemic and kept so by bleeding, and the rate of production of haemoglobin was estimated by the amount of haemoglobin which it was necessary to remove in order that the animals should be kept at a constant level of anaemia, according to the method used by Whipple and Robscheit-Robbins (1925).

Methods of Present Experiment

The dogs used were adult mongrels of the foxhound type, weighing 13.7 and 13.75 kg. respectively. They were fed a constant diet of Gaines dog food* with no additions, and remained in good health throughout the time of the experiment. Free acid was present in a fasting specimen of stomach contents from each dog.

Blood counts were performed on venous blood removed by venepuncture with little or no stasis from the jugular or femoral veins. Red cell counts were performed by the usual methods and the percentage of haemoglobin was estimated by means of a Sahli haemoglobinometer so calibrated that 100 per cent. of haemoglobin was equivalent to 13.8 grammes of haemoglobin per 100 c.cm. of blood. The mean corpuscular volume was calculated from the red cell count and the percentage volume of packed cells, as determined by centrifuging a sample of blood for twenty minutes in a Win-trobe haematocrit tube. Reticulocyte counts were made by the dry cover-slip method, the reticulocytes present in 500 red cells being counted. In the first eight days blood was removed by venepuncture till the animals' haemoglobin level was between 40 and 50 per cent. Throughout the rest of the experiment blood was removed once or twice a week in amounts sufficient to keep the haemoglobin percentage at this level.

Iron was administered in two ways in the periods shown on the chart. It was given by mouth as reduced iron in gelatin capsules in daily doses of 1 gramme. The capsules were placed on the back of the dogs' tongues and a few cubic centimetres of water were then poured into their mouths to induce them to swallow. Iron was also given by subcutaneous injection in the form of daily injections of 20 mg. of iron and ammonium citrate in aqueous solution. Pure bilirubin (Eastman Kodak) was given intravenously in doses of 50 mg. daily, dissolved in 10 c.cm. of hot decimolar sodium carbonate solution.

* Gaines dog food contains, according to its makers, beef, skimmed milk, egg yolk, beets, soya beans, whole wheat, yellow corn, rice, malt syrup, bone meal, calcium carbonate, cod-liver oil, and carotene. Analysis by weight: protein 23 per cent. (minimum), fat 3 per cent. (minimum), fibre 4 per cent. (maximum), carbohydrate 50 per cent. (minimum). Each dog was given approximately 1½ lb. a day of the dry material.

Table showing Effect of Repeated Intravenous Injections of Bilirubin on Rate of Production of Haemoglobin in Dogs kept Anaemic by Bleeding

Period		Length of Period in Days	Dog 603		Dog 605	
			Total Hb removed in Grammes	Average Hb removed per Day in Grammes	Total Hb removed in Grammes	Average Hb removed per Day in Grammes
I	Bleeding down to anaemia level	10	188	18.8	192	19.2
II	Reticulocytosis	20	60	3.0	54	2.7
III	Control period	20	45	2.3	51	2.6
IV	Reduced iron 1 gramme daily by mouth	25	63	2.5	89	3.6
V	Iron and ammonium citrate 20 mg. daily by injection	21	74	3.5	117	5.6
VI	Reduced iron 1 gramme by mouth	20	122	6.1	118	5.9
VII	Reduced iron, 1 gramme by mouth and bilirubin 50 mg. intravenously daily	22	171	7.8	155	7.1
VIII	Reduced iron 1 gramme by mouth	27	170	6.3	159	5.9
IX	Control period	19	77	4.1	65	3.4
X	Control period	22	33	1.5	23	1.0
XI	Bilirubin 50 mg. intravenously daily	18	37	2.1	21	1.2
XII	Bilirubin 50 mg. intravenously and reduced iron 1 gramme by mouth daily	14	52	3.8	35	2.5

Chart showing effect of repeated injections of bilirubin on percentage of reticulocytes and rate of production of haemoglobin in two dogs kept anaemic by bleeding.

Results

The results obtained are shown in a chart and in a table, in which the time of the experiment has been divided into periods numbered I to XII. In Period I, lasting ten days, the two animals were bled down to a level of haemoglobin of 40 to 50 per cent., the average daily amount of haemoglobin removed being 18.8 and 19.2 grammes respectively. In Period II, lasting twenty days, which includes the subsequent reticulocyte response, the amount of haemoglobin removed each day was 3 and 2.7 grammes. Period III, lasting twenty days, is taken as a control period, during which the average daily amount of

haemoglobin removed was 2.3 and 2.6 grammes. In Period IV, lasting twenty-five days, iron was given by mouth in the form of reduced iron in doses of 1 gramme daily. The average daily amount of haemoglobin removed increased slightly to 2.5 and 3.6 grammes respectively, but no significant increase in the percentage of reticulocytes was observed. During Period V, lasting twenty-one days, iron was given by subcutaneous injection in the form of iron and ammonium citrate in doses of 20 mg. daily. The average daily amount of haemoglobin removed increased to 3.5 and 5.6 grammes respectively, and reticulocyte responses, with peaks of 8 and 13 per cent. respectively, occurred in each dog. In Period VI, lasting twenty days, the animals were again given reduced iron by mouth. Three days after the beginning of this period they were given in addition daily intravenous injections of 50 mg. of pure bilirubin dissolved in 10 c.cm. of decimolar sodium carbonate. After two injections there was

a sharp rise in the reticulocyte count. The significance of this rise was doubtful, and injections of bilirubin were therefore discontinued for the remainder of this period in order to eliminate the possibility that the reticulocyte responses were due to some cause other than the administration of bilirubin. Periods V and VI therefore serve to show that a raised reticulocyte count could not be maintained by the administration of iron alone, given either by injection or by mouth. During Period VII, lasting twenty-two days, the administration of iron by mouth was continued together with daily intravenous injections of 50 mg. of bilirubin. There occurred a prompt reticulocyte response, the percentage of reticulocytes rising to the highest figures (10 and 15 per cent.) recorded for each dog throughout the experiment, and remaining above normal for seventy to eighty days and long after the administration of bilirubin had been discontinued. The daily average amount of haemoglobin removed rose to the highest figures recorded—7.8 and 7.1 grammes respectively. In Period VIII, lasting twenty-seven days, the administration of iron by mouth was continued, but the injections of bilirubin were discontinued. The reticulocyte count fell slowly, but remained considerably above normal, and the average daily amount of haemoglobin removed decreased to 6.3 and 5.9 grammes respectively. In Period IX, lasting nineteen days, neither iron nor bilirubin was administered; the reticulocyte count decreased, but remained above normal, and the daily average amount of haemoglobin removed was 4.1 and 3.4 grammes respectively. During Period X, which is taken as a further control period, the reticulocyte count returned to normal and the daily average amount of haemoglobin removed was 1.5 and 1 gramme respectively. In Period XI, lasting eighteen days, bilirubin was administered as before, but no iron was given. On this occasion there was a small reticulocyte response (to 4 and 6 per cent. respectively), and the average daily haemoglobin removed increased slightly to 2.1 and 1.2 grammes respectively. During Period XII, lasting fourteen days, the daily injections of bilirubin were continued, and in addition iron was administered by mouth as before. There was a further reticulocyte response (to 7 per cent. in each dog), and the daily average amount of haemoglobin removed increased to 3.8 and 2.5 grammes respectively.

The changes which took place in the mean corpuscular volume are recorded in the chart but will not be further discussed. A decrease in the colour index (indicated by divergence of the lines representing red cell count and percentage of haemoglobin) occurred in periods of iron deficiency, and the reverse was experienced in periods in which iron was administered for any considerable time.

Discussion

The experiment shows that repeated intravenous injections of pure bilirubin had a considerable effect on erythropoiesis and on the production of haemoglobin in two dogs kept anaemic by bleeding. This effect consisted of a prompt reticulocyte response, which lasted for some seventy days and continued for some fifty days after the injections of bilirubin had been discontinued, and an increase in the amount of haemoglobin that had to be removed to keep the animals at a constant level of anaemia. The differences between the amount of haemoglobin removed daily in Periods VI and VII may appear small in the chart, but they actually represent increases of 20 and 28 per cent. respectively in the amount of haemoglobin produced by the two dogs. It must also be remembered that two injections of bilirubin had been given to each dog early in Period VI; and as they brought about a noticeable reticulocyte response they may also have caused some increase in the amount of haemoglobin produced in this period.

It does not seem wise to do more than speculate as to the nature of the mechanism of this effect of bilirubin injections on the production of haemoglobin. Comparison of Period IV (iron by mouth without bilirubin) with Periods VII and XII (iron by mouth with bilirubin) suggests that the bilirubin acted by improving in some way the absorption or the utilization of iron. This suggestion,

however, requires close scrutiny, for though on a given amount of iron by mouth haemoglobin production was much greater in Period VII (with bilirubin) than in Period IV (without bilirubin) the periods are not comparable; at the beginning of Period IV the animals were in a state of iron deficiency, but this was not the case at the beginning of Period VII, when they had been receiving iron in some form for seventy days. Periods IV and XII are more comparable, since in each case the animals had received no iron for some fifty days. Though the average daily amount of haemoglobin produced by the two dogs is about the same in these two periods the increase in the amount of haemoglobin produced in Period XII over that produced in Period XI (81 and 108 per cent. respectively) was much greater than the increase in Period IV over that in Period III (9 and 39 per cent. respectively). Moreover, in Period XII there was a significant reticulocyte response, whereas in Period IV there was none. There is therefore some evidence to suggest that bilirubin given by intravenous injection improves the absorption or the utilization of iron.

Whether or no this is the case, comparison of Period VII with Period XI indicates that iron deficiency limits the effect of bilirubin; for in Period VII (administration of iron and no previous iron deficiency) the effect of a given amount of bilirubin on the reticulocyte count and on the production of haemoglobin was far greater than in Period XI (administration of bilirubin without addition of iron to animals in a state of iron deficiency).

A simple explanation of the findings would be that after repeated bleedings the animals were short of unknown pigment precursors of haemoglobin as well as of iron, and that the bilirubin could be utilized to fill this deficiency. If this was the case administration of either substance might be expected to reinforce the effect of administration of the other. As discussed, however, in the introduction to this paper, the experiments of Hawkins *et al.* on the effects of injections of haemoglobin make the supposition that injected bilirubin is actually utilized in the formation of new haemoglobin seem improbable. The question could be settled by repeating the experiment reported here on dogs with bile-renal fistulae, in which accurate estimations of the amount of pigment excreted can be made.

The suggestion that bilirubin acts as an erythropoietic hormone—that a product of the destruction of haemoglobin stimulates increased activity of the bone marrow and the formation of new haemoglobin—is an attractive but as yet hypothetical one.

Summary

An experiment was made on two anaemic dogs to discover the effect of repeated intravenous injections of pure bilirubin on the rate of production of haemoglobin, the size of the red cells, and the reticulocyte count.

It was found that the injections of bilirubin caused an increase in the rate of production of haemoglobin and a prolonged reticulocyte response.

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REFERENCES

- Fellinger, K. (1932). *Z. ges. exp. Med.*, **85**, 369.
Hawkins, W. B., Sribhishaj, K., Robschtein-Robbins, F. S., and Whipple, G. H. (1931). *Amer. J. Physiol.*, **96**, 463.
— and Johnson, A. C. (1939). *Ibid.*, **126**, 326.
McMaster, P. D., and Haessler, H. (1921). *J. exp. Med.*, **34**, 579.
Miller, D. K., and Rhoads, C. P. (1934). *Ibid.*, **59**, 333.
Patek, A. J., and Minot, G. R. (1934). *Amer. J. med. Sci.*, **188**, 206.
Verzar, F. (1929). *Z. ges. exp. Med.*, **63**, 475.
Whipple, G. H., and Robschtein-Robbins, F. S. (1925). *Amer. J. Physiol.*, **72**, 395.
Zih, A. (1939). *Z. ges. exp. Med.*, **104**, 675.