# AN EXAMINATION OF A SUGGESTED LABORATORY METHOD OF ASSAY OF THE ANTI-PERNICIOUS ANÆMIA FRACTION OF LIVER EXTRACTS

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Since the clinical method of testing the potency of liver extracts is a lengthy and only roughly quantitative procedure, a laboratory method of assay would be advantageous. Watson, Cameron and Witts[1] described the incidence of a macrocytic anæmia resembling human Addisonian pernicious anæmia in albino rats in which a blind loop, tending to fill by peristalsis, had been made in the small intestine. The anæmia responded to treatment with liver extract: it seemed possible, therefore, that this technique could be used to develop an assay method. The following experiments were undertaken with this object in view.

The operation described by Watson, Cameron and Witts (*loc. cit.*), was performed on 15 female and 89 male albino rats. These rats and 28 control animals (10 female and 18 male) were maintained on the diet given in Table I.

TABLE I

Diet

| | |
|---|---|
| Casein (light white) | 25 per cent. |
| Rice Starch | 68 „ „ |
| Cod-liver oil | 2 „ „ |
| Arachis oil | 2 „ „ |
| Salt mixture | 3 „ „ |

Vitamin supplements

(Administered mixed with a small amount of basal diet)

| | per 20 rats |
|---|---|
| i. Daily | |
| Aneurine hydrochloride | 2 mg. |
| Riboflavine | 4 mg. |
| Nicotinic acid | 20 mg. |
| Pyridoxine | 2 mg. |
| Calcium pantothenate | 4 mg. |
| Menaphthone (Prokayvit) | 0·8 mg. |
| Choline chloride | 200 mg. |
| ii. Twice weekly | |
| Vitamin A (Radiostoleum) | 30,000 I.U. |
| Vitamin D( „ „ ) | 6,000 I.U. |
| iii. Once weekly, during early stages of experiment only:— | |
| α-Tocopherol | 60 mg. |

Since we were concerned with the possibility of routine assay work rather than with the nature of the anæmia developed, full hæmatological studies were not undertaken, but weekly determinations of erythrocyte and reticulocyte counts and of hæmoglobin levels were made. A group of 20 female rats was first used, the survivors being sacrificed after 3 months. Operations were then performed on a larger number of male rats, some of which survived for a period of 12 months or more. Table II, column (a) gives the results of 104 operations.

TABLE II

| Cause of death after operation | (a) | (b) |
|---|---|---|
| Under anæsthetic .. .. .. | 4 | — |
| Rupture of anastomosis .. .. .. | 40 | 6 |
| Obstruction .. .. .. .. | 14 | 1 |
| Sepsis .. .. .. .. .. | 12 | 2 |
| Anæmia .. .. .. .. .. | 6 | — |
| Anæmia with liver necrosis .. .. | 1 | — |
| Not known .. .. .. .. | 8 | 2 |
| Killed owing to condition .. .. .. | 9 | 2 |
| Survived .. .. .. .. .. | 10 | 2 |

This summary includes a group of 15 male rats (b) which when received were heavily infected with a *Salmonella* type of organism; 3 doses of 30 mg. sulphaguanidine per rat orally, prior to operation, cleared them of the infection. Deaths under the anæsthetic were found to be due to an unsuspected infection of the lungs. Intestinal obstruction occurred in 5 animals within a few weeks of the operation, in 3 in from 13 to 23 weeks. It was accompanied by septic conditions in 3 and by a marked fall in the red blood cell counts and hæmoglobin levels in 6. 9 rats were killed owing to their bad condition. There was a fall in the red blood cell counts and hæmoglobin levels in 4 of the rats in which sepsis occurred.

The most serious single factor causing death was rupture of the anastomosis, such fatalities occurring within a few days of operation. Variation of the method of anastomosis did not appear to affect the percentage of successful operations. On several occasions, the rats were starved prior to operation, in the hope that rupture would be less likely to occur in the absence of food passing the anastomosis, but the mortality rate was not reduced. Poth *et al.*[2] have reported the beneficial effect of sulphasuccidine on wound healing in end-to-end anastomosis of the descending colon in dogs. No reduction in immediate mortality was observed in our group of rats which had received sulphaguanidine preoperatively, but there were no deaths from obstruction in the first 4 weeks after operation. It is possible that the anastomoses healed with the formation of less constricting scar tissue, which would be likely to cause obstruction. Cameron, Watson and Witts[3] obtained a 70 per cent. survival from the immediate effects of the operation. Our lower percentage is thought to be due, not to difference of technique, but to our use of smaller rats.

A further difficulty in the earlier operations was the high rate of incidence of infection of the wound scar. Large abcesses occurred at either end of the wound, and, if not resulting in death, necessitated frequent treatment and seriously affected blood counts. This complication was completely prevented by the use of iodine for pre-operative sterilisation of the skin and of catgut for suture of the peritoneum and muscles, instead of flavine and thread respectively as recommended by Witts[4]. The incidence of other septic conditions (e.g., internal abscess, abscess of the diverticulum) was not affected by this alteration in technique.

The erythrocyte and reticulocyte counts and hæmoglobin levels of the rats were determined weekly for 2, 3 or 4 weeks prior to operation. When first placed on the experimental diet, different batches of rats had different average blood levels, but they became more uniform after a short time under standard dietary conditions. Control rats which were examined parallel with the operated rats showed marked fluctuations in erythrocyte counts, although the hæmoglobin level remained fairly constant, with a slight downward trend over a period of 6 months. Only a small number of controls were retained for the full duration of the experiment and the poor condition of one or two of those remaining after 6 months markedly lowered the average hæmoglobin and erythrocyte values. The mean values for both showed several pronounced fluctuations coincidently in operated and control rats due presumably to environmental changes. Cameron and Watson[5] report an average erythrocyte count of 8·50 million/c.mm. and an average hæmoglobin level of 14·6 g./100 ml. for adult male albino rats maintained on a synthetic diet. The average values obtained in this laboratory were an erythrocyte count of 7·89 million/c.mm. and a hæmoglobin level of 12·2 g./100 ml. Cameron, Watson and Witts (*loc. cit.*) diagnosed anæmia if the hæmoglobin level fell below 10 g./100 ml. Our controls fell occasionally below this limit, but the fall was transient except in the one or two unhealthy animals referred to above. A fall below 10 g. was, therefore, taken as an index of anæmia. A drop in erythrocyte count below 6 million/c.mm. was generally indicative of anæmia, but the wide fluctuations seen in normal animals made this an unreliable criterion. The red cell count of anæmic rats has been recorded as low as 1·60 million/c.mm., but this degree of anæmia was too severe for recovery. A fall in erythrocyte count below 4·1 million/c.mm., or in hæmoglobin level below 7·3 g./100 ml., was always followed by death. The fall in hæmoglobin level sometimes lagged behind the drop in erythrocytes and *vice versa*, while often either hæmoglobin or erythrocyte level alone was low. Both values were usually low if the anæmia was fatal or severe.

57 rats survived one or more weeks after operation, including 9 in the group treated with sulphaguanidine. 32 of the former group died after an average of 11·4 weeks (1 to 41 weeks) and 12 were killed after 13·3 weeks (4 to 35 weeks): two survived 43 weeks and two 61 and 62 weeks respectively. Of the group treated with sulphaguanidine, 5 died (1 to 23

weeks), 2 were killed (17 to 34 weeks) and 2 were still living after 34 weeks.

A number of animals developed an anæmia of short duration, usually with spontaneous remission, within 2 or 3 weeks of operation. This type of anæmia has not been included in the classification of results given above. It occurred in 2 (25 per cent.) of the rats which received sulphaguanidine, and in 26 (55 per cent.) of the remainder. The 26 rats developed anæmia at an average interval of 2½ weeks after operation, all but 6 showing spontaneous recovery. Of these 6 rats, 4 did not recover: one had septicæmia, one had a septic wound and an intestinal obstruction, and the remaining 2 were not examined post-mortem. One of the latter had not responded to doses of 0·2 ml. and 0·8 ml. of anahæmin. Two rats, however recovered after treatment with anahæmin, 1 receiving 0·45 ml., the other 0·2 ml. and repeated doses of 0·8 ml. The 2 rats in the group which had sulphaguanidine recovered from their post-operative anæmia.

Of the 57 animals which survived one or more weeks after operation, 28 (49 per cent.) developed anæmia after a period ranging from 6 to 45 post-operative weeks. In addition to these rats, one animal which had only partially recovered from post-operative anæmia relapsed at 4 weeks and repeatedly became anæmic; another which was anæmic 2 weeks after operation and recovered, relapsed at 5 weeks and recovered, relapsing again at 15 weeks. Excluding these rats in which there is no clear differentiation of post-operative from subsequent anæmia, the average time elapsing before anæmia developed was 13 weeks.

These results are comparable with those of Cameron, Watson and Witts (*loc. cit.*), who found macrocytic anæmia in 42 per cent. of animals surviving the immediate effects of operation. Anæmia developed after an average interval of 10 weeks, the length of the interval ranging from 4 to 20½ weeks.

The fate of the rats treated with sulphaguanidine is of particular interest since the drug will presumably have influenced the intestinal flora. The development of anæmia after formation of a blind loop in the small intestine may be due to a change in the flora of the loop and intestine. In view of this possibility it is interesting to note that no case of sustained anæmia occurred in this group of rats, except coincidentally with abscess formation.

*Response to Anahæmin**.—The anahæmin was administered intramuscularly into the hind legs of the anæmic rats. The response was extremely variable. In animals suffering only from anæmia, remission was often spontaneous. Satisfactory responses to doses of anahæmin of 1·6 to 2·6 ml./wk. were observed. These animals survived. A satisfactory response early in life may not be repeated later, when the response to heavier dosage may be unsatisfactory. The condition of the blood may improve, but death ensue. Vitamin $B_{12}$, 30μg., was given in one case of severe anæmia without response. In other anæmic rats, no response to

repeated doses of up to 2·4 ml./week of anahæmin was seen. These animals were killed later in poor condition. In cases of obstruction, there was usually an unsatisfactory response to heavy dosage (up to 3·6 ml./week), except in 2 rats to doses of 0·45 and 1·6 ml./week respectively in the earlier stages of recurrent anæmia; spontaneous remission occurred in similar instances. The response was not repeated on subsequent relapse. In animals suffering from sepsis, there was no response to doses of 0·45 to 3·6 ml./week.* In addition to the above results, it was found that a low or moderate dose of anahæmin may cause a rise in the red blood cell count and hæmoglobin level in a non-anæmic rat.

The condition, after operation, of two rats, one of which was anæmic, and of 2 control animals, became poor with swelling and fissuring of the paws and some loss of hair. 10 mg. of pyridoxine was given to each by intraperitoneal injection. Hæmoglobin and erythrocyte levels rose in the rats which had been operated upon, in the non-anæmic one reproducing a previous response to anahæmin, except that, after pyridoxine, the rise in hæmoglobin level was more pronounced. The anæmic rat received anahæmin concurrently with the pyridoxine. A satisfactory hæmoglobin response followed this treatment where two or three weeks previously the hæmoglobin had not fully responded to anahæmin alone. Injection of pyridoxine was followed in the control rats by a marked rise in hæmoglobin level, and a sharp temporary increase in erythrocytes. The external appearance of all four animals slowly improved, although the pyridoxine treatment was not repeated.

Watson, Cameron and Witts' original statement (*loc. cit.*) that their anæmic animals could be kept alive by treatment with liver extract was not supported by their further experiments (Cameron, Callender, Watson and Witts[6]). They suggested[6] that the unsatisfactory response was due to the fact that the condition induced by operation is not a simple vitamin deficiency. The life of some of our animals was apparently prolonged for many weeks by treatment with anahæmin, but it is impossible to decide how far this survival was due to the treatment and how much to spontaneous remission of the anæmia. It proved impossible to relate the dose of anahæmin to the extremely variable reticulocyte response. A reticulocyte response to anahæmin was observed in both control and operated rats.

## Conclusion

This method appears unsuitable for the development of any routine test of the potency of liver extracts. The reticulocyte response is too variable to be a reliable criterion of potency, and repeated bleeding to follow this response has a deleterious effect on the animals. Hæmoglobin levels and erythrocyte counts do not respond in any uniform manner and are readily affected by other factors. In any case, it is impossible to determine until after death whether or not the anæmia is complicated by infection or by obstruction of the intestinal tract. From a practical stand-

* All batches of anahæmin have been found potent by clinical test.

point, it may be added that the wastage of animals in high development of anæmia and relapse after treatment are often slow and determination of blood levels on any useful scale extremely laborious.


## SUMMARY

1. A blind loop was made in the small intestine of rats, in such a manner that it tended to fill by peristalsis, according to the method described by Cameron, Watson and Witts.

2. 15 female and 89 male rats were operated on: the immediate post-operative mortality was 43 per cent. (chiefly rupture of the anastomosis).

3. 28 rats developed anæmia within 2 to 3 weeks of operation, 22 of which recovered spontaneously from this anæmia.

4. 28 rats developed anæmia from 6 to 45 weeks after operation.

5. The effect of injection of anahæmin in anæmic and non-anæmic rats is described.

6. The value of this method as a method of assay of liver preparations is discussed. It is concluded that it is not a practicable method, since it does not provide a reliable criterion of the potency of clinically active liver extract (anahæmin).


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## REFERENCES


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