XI. STUDIES ON BAYER 205 (GERMANIN) AND ANTRYPOL[1]

III. FURTHER OBSERVATIONS ON THE METHOD OF DETERMINATION AND ON THE RETENTION OF THIS DRUG IN THE ANIMAL BODY

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In a previous communication [Dangerfield *et al.* 1938] a method for the determination of Bayer 205 and Antrypol in the blood plasma was described, and experimental evidence was produced to show that after the injection of a "normal" dose of Bayer 205 into a rabbit or dog there is a significant amount of the drug in the plasma 3 months or even longer after the injection. Further investigations have been concerned (*a*) with the simplification of the method (by the use of a Lovibond comparator) so that Bayer 205 determinations on blood plasma, C.S.F. and possibly other body fluids might be possible in tropical countries when a colorimeter is not available, and (*b*) with a modification of the method for determinations on urine. The observations on the retention of the drug in the body have also been extended in order to provide a continuous curve to show the amount of Bayer 205 in the plasma of rabbits during and after a series of injections of the drug. During these investigations no appreciable difference has been observed between Bayer 205 and Antrypol, and in most of the later experiments the latter preparation has been used.

Method of determination

It has not been found necessary to make any significant alteration in the method described in the earlier paper, but one or two slight improvements have been introduced. The hydrolysis by conc. HCl is now effected in glass-stoppered pyrex tubes graduated at the 10 ml. level. At one period in these investigations difficulty was experienced in matching the final colours, owing to occasional development of turbidity following the addition of the methyl-α-naphthylamine solution. It was found that this turbidity was due to impurities (possibly Cu) in the distilled water, and this trouble was overcome by passing the distilled water twice through a permutit water-softener.

Subsequent work has fully confirmed the suggestion that methyl-α-naphthylamine hydrochloride has many advantages over the free base when used as the coupling agent. The free base (an oil) develops a red colour fairly rapidly whereas the solid hydrochloride shows very little change in colour over a long period, particularly if exposure to sunlight is reduced to a minimum. The solution used in the actual determination (0·2 g. in 100 ml. in 50 % v./v. acetic acid) shows very little reddening in 1 or 2 weeks if kept in a dark bottle.

[1] This communication represents a second report to the Medical Research Council at whose request this work was undertaken.

Preparation of methyl-α-naphthylamine hydrochloride. The preparation of the free base was essentially that described by Fischer [1895]. The following additional details of the method might prove useful. α-Naphthylamine was formylated [Tobias, 1882], and the product recrystallized and then methylated. For the methylation the xylene should be perfectly dry and granulated sodium should be used. After the addition of the sodium, a vigorous reaction occurred and a white precipitate formed; the mixture was cooled to 80°, methyl iodide added and the temperature raised to 100–110°, with subsequent completion of the reaction by boiling the mixture under reflux for 1 hr. The cooled product was filtered and the filtrate was treated with charcoal and freed from xylene by steam distillation. The formylmethyl-α-naphthylamine was hydrolysed by boiling for 1 hr. under reflux with 5 vol. dil. H_2SO_4 (10% v./v.), the solution filtered and treated with charcoal, and the final filtrate kept overnight, when a little α-naphthylamine separated out. The filtered solution was basified with NaOH, and the base extracted with ether. The ethereal solution was treated with charcoal and dried with $CaCl_2$. Dry HCl was passed into this solution to precipitate methyl-α-naphthylamine hydrochloride. The precipitate obtained in the early stages contained some violet impurity and was discarded. The main bulk precipitated by further passage of HCl was dried *in vacuo* in the presence of solid KOH. No further purification by recrystallization was possible but this preparation, which had only a very faint pink tinge, was pure enough for the purposes of this investigation. The yield was 12·8 g. of the hydrochloride from 14 g. of crude base, and a similar yield was obtained from a sample of the base supplied by Messrs May and Baker, Ltd., Dagenham.

The use of a Lovibond comparator. At the suggestion of Dr F. Hawking, who wished to carry out determinations of Bayer 205 in East Africa, coloured discs have been made for this purpose by "The Tintometer Ltd." A set of two discs covers the ranges equivalent to 0·18 to 1·20 and 1·20 to 3·60 mg. of hydrolysed Bayer 205 per 100 ml. of solution to be diazotized. The matching of the colours is quite easy and can be done with accuracy up to the reading of 2·70. With larger amounts of Bayer 205 it is advisable to use a smaller volume (i.e. less than 2 ml.) of the hydrolysed solution and to add 3 *N* HCl to give 2 ml. for the diazotization process. When these discs are used it is possible to determine with satisfactory accuracy amounts of Bayer 205 greater than 0·5 mg./100 ml. of plasma or other fluid. On the rare occasions when smaller amounts have to be determined, the solution should be compared with a set of standards.

The comparator method has several advantages over the colorimeter method. Normal plasma and sera contain a small amount of diazotizable substances which can easily be allowed for by the use of the control tube, and this compensation for "blank values" has proved of considerable value for the determination of Bayer 205 in urine and certain animal tissues. When a "blank" of this type is not available, compensation for any yellow or brownish colour in the filtered hydrolysates can be made by using, as a control, a mixture of the filtrate and all the other reagents except the sodium nitrite.

Products of acid hydrolysis of Bayer 205

Lang [1931] states that the hydrolysis of Bayer 205 by hot HCl yields *m*-aminobenzoic acid, *m*-amino-*p*-toluic acid and α-naphthylamine-4:6:8-trisulphonic acid, but there appears to be no evidence that the disruption of the complex molecule of the drug is as complete as this. For another aspect of our investigations these three amines are required, and the opportunity has been taken to determine whether a mixture of the three would give the same colour

when diazotized and coupled with methylnaphthylamine as does an equivalent amount of hydrolysed Bayer 205. The colour obtained with an equimolecular mixture of these amines was approximately as intense as, but definitely more purple than, the colour with hydrolysed Bayer 205. The results suggest that a large part of the colour with the last-named is due to *m*-aminobenzoic acid. Each of the other two amines gives a lilac colour and these observations tend to show that hydrolysis with HCl is not as complete or simple as was suggested by Lang. This point is perhaps of theoretical interest only at present, and does not affect in any way the method of determination of Bayer 205, but it may be of significance when the possible decomposition of this drug in the body is studied.

Determination on serum

For some purposes it may be necessary to use serum for these determinations, and a comparison has been made of the values obtained with the plasma and sera of rabbits which had previously received injections of Antrypol. Blood was collected from the ear of each rabbit in amounts of 2·5, 5·0 and 2·5 ml. The first and third samples were collected in oxalated tubes and were then mixed and centrifuged for the separation of the plasma. The second blood sample was allowed to clot and the serum obtained. Bayer 205 determinations were made, and the results recorded in Table I show that the plasma and serum from blood containing Bayer 205 contain the same amount of this drug.

Table I. *Bayer 205 (or Antrypol) determinations on plasma and serum*

	mg. Antrypol per 100 ml.	
	Plasma	Serum
Rabbit 493 (Antrypol)	3·50	3·60
,, 494 (Antrypol)	3·40	3·40
,, 495 (Control)	0·95	1·00
,, 496 (Control)	0·95	0·95

The results in this table have not been corrected for the blank value of approximately 1·0 mg./100 ml. for normal rabbit plasma or serum.

Determinations on urine

In an earlier paper [Dangerfield *et al.* 1938] it was mentioned that the method devised for the determination of Bayer 205 in plasma cannot be used for determinations of the drug in urine because of the presence in normal urine of a very variable amount of diazotizable substances. The necessary blank values may be as low as 0·25 or as high as 5·0 mg./100 ml. (calculated as hydrolysed Bayer 205), and it is not possible by any simple method to compensate for this blank value. There is no satisfactory evidence as yet that unchanged Bayer 205 is excreted in the urine, but the excretion in the urine of a trypanocidal agent after the injection of the drug [Mayer & Menk, 1921; 1922; Thomson & Robertson, 1922] and some preliminary observations recorded earlier in the present investigations [Dangerfield *et al.* 1938] suggest that this excretion does take place. Further investigations which will be reported later leave no doubt that Bayer 205 either in the original or slightly modified form, is excreted in the urine in fairly appreciable amounts for a few days after the injection.

Since the use of a blank value for normal urine is precluded by the wide variation in this value, efforts have been made to destroy the "normal" amines without loss of Bayer 205, but without success. Ultimately it was found possible, however, to separate these normal amines (or "amine-precursors") from Bayer

205 by ultrafiltration through a standard collodion membrane, and this is the basis of the method now adopted. Collodion membranes are prepared by the method described by Folley [1933] and are dried for 15 min. in a current of air passing though the apparatus at the rate of 2 l. per hr. at room temperature (19–21°). The membranes are then immersed in distilled water for 24 hr., and filtration is effected with a positive pressure of 120 mm. Hg in the apparatus described by Folley. Normal urine (of man or dog) yields an ultrafiltrate which, on hydrolysis with HCl, gives the same amount of diazotizable amine as does the untreated urine, but no Bayer 205 passes through the membranes. If determinations are made, therefore, on a urine containing Bayer 205 and on an ultrafiltrate from this urine, the difference between the two values is a measure of the Bayer 205 present.

The "recovery" of Bayer 205 when added to urine is, however, not quantitative, but usually varies between 70 and 95% with an average of about 85%. (With one sample of urine, however, the recovery was a little below 50%.) This loss is fairly constant for any given sample of urine, e.g. with the same urine the loss may be 15% with amounts of Bayer 205 varying from 1 to 10 mg./100 ml. (cf. Table II). A fairly satisfactory estimate of the true Bayer 205 content of the urine can thus be obtained by the use of a Bayer 205 "recovery factor" for the urine concerned, this factor usually being between 1·10 and 1·25. For a true estimate of the amount of Bayer 205 in any sample of urine it is necessary therefore, to carry out determinations on (*a*) the urine, (*b*) an ultrafiltrate from the urine, prepared as described above and (*c*) the urine treated with a known amount of Bayer 205 (e.g. 2 or 3 mg./100 ml. of urine). If (*c*) is prepared by the addition of a small amount of strong Bayer 205 solution to the urine, the urine for (*a*) and (*b*) should be diluted with a corresponding amount of water. The difference between (*a*) and (*b*) will give the minimum Bayer 205 content of the urine (or diluted urine), and a more correct value is obtained by multiplying this value by a factor if the difference between (*a*) and (*c*) shows that the recovery is not complete. With the Lovibond comparator method these differences can be measured very readily since the less-coloured sample can be placed in the control tube. (A control determination is, of course, made on the Bayer 205 solution used for the preparation of (*c*), and in all the experiments quoted here duplicate determinations were made.)

Several possible explanations for this incomplete recovery of Bayer 205 added to urine have been examined, in the hope that some slight modification might render the use of a recovery factor unnecessary. A longer period of hydrolysis (8 hr. instead of 6 hr.) or an increase in the amount of HCl, does not increase the yield. One of the factors concerned is possibly the presence in hydrolysed urine of substances which prevent the maximum colour development, e.g. the liberation of phenolic substances from ethereal sulphates and the combination of these phenols with the diazotized amines. The addition of hydrolysed urine to a separately hydrolysed Bayer 205 solution causes only a slight loss of amine (about 5%), and this explanation can account for only part of the total loss. It was ultimately decided that it is wiser to adopt a correction factor in these investigations rather than attempt to devise a complicated technique which may lead to greater inaccuracies.

Some of the results of determinations on normal urines after the addition of Bayer 205 are recorded in Tables II and III, and from these results it will be seen that the method can be used for the detection and determination of small amounts of Bayer 205 or Antrypol. Amounts greater than 0·6 mg./100 ml. of urine can be determined with satisfactory accuracy. A chemical method of this type cannot,

Table II. *Recovery of Bayer 205 added to normal urine*

Subject	Amount of Bayer 205 added	"Amine" plus Bayer 205	Bayer 205	Recovery %
	mg. Bayer 205/100 ml.			
1	0	0·66	—	—
	1·0	1·48	0·82	82
	2·0	2·33	1·67	83
	3·0	3·07	2·41	80
	5·0	4·67	4·01	80
2	0	1·93	—	—
	0·97	2·66	0·73	75
	3·10	4·33	2·40	77
3	0	0·70	—	—
	0·95	1·60	0·90	95
	3·0	3·27	2·57	86
	11·0	10·0	9·30	85
4	0	0·77	—	—
	1·0	1·59	0·82	82
	3·2	3·33	2·56	80
	10·7	10·20	9·43	88
5	0	0·67	—	—
	1·00	1·60	0·93	93
	3·0	3·43	2·76	92
	10·3	10·40	9·73	94
6	0	0·73	—	—
	1·00	1·20	0·47	47
	2·20	1·62	0·89	40
	4·50	2·68	1·95	43
	7·85	4·13	3·40	43

The above results are average values of two or three determinations (i.e. separate hydrolyses). The urines for these determinations and for those given in Table III were diluted with one-ninth their volume of water or Bayer 205 solution (of ten times the strength recorded in column 2).

Table III. *Determination of Bayer 205 (or Antrypol) in urine*

Amount of Bayer 205 added to urine	Bayer 205 plus "amine": Untreated urine	Bayer 205 plus "amine": Urine ultrafiltrate	Bayer 205 value	Corrected* Bayer 205 value
2·0	2·59	0·98	1·61	2·07
0	1·61	1·60	—	—
1·0	2·47	1·55	0·92	1·13
3·0	4·00	1·57	2·43	2·99
0	1·80	1·78	—	—
1·0	2·50	1·72	0·78	1·08
3·0	4·00	1·80	2·20	3·04
0	0·25	0·25	—	—
1·86	1·80	0·25	1·55	1·89
3·95	3·33	0·25	3·08	3·76
5·87	5·08	0·25	4·83	5·89
0	0·72	0·70	—	—
2·17	2·59	0·72	1·87	2·19
4·43	4·60	0·70	3·90	4·56
6·67	6·40	0·72	5·68	6·65

* Obtained by multiplying the figure in the previous column by a factor which is calculated from values obtained with mixtures of the untreated urine and known amounts of Bayer 205. This factor is usually between 1·10 and 1·25.

All the figures in the above Table represent mg./100 ml.

of course, differentiate between Bayer 205 and similar substances which might be produced from this drug in the animal body. The use of this ultrafiltration method for urine probably ensures, however, that relatively simple substances produced from Bayer 205 will not be estimated with the parent substance since the membranes used are completely permeable to *m*-aminobenzoic acid. *m*-Amino-*p*-toluic acid and α-naphthylamine-4:6:8-trisulphonic acid, the other amines which, according to Lang [1931], are produced by acid hydrolysis of Bayer 205, are retained by the membranes to a slight extent only.

Retention of Bayer 205 in the plasma following injection of the drug

A preliminary investigation to determine the length of time during which injected Bayer 205 could be detected in the plasma of rabbits following a single or a series of injections of the drug was described in an earlier paper [Dangerfield *et al.* 1938]. The results obtained suggested that a series of injections of small amounts results in a more satisfactory maintenance of a significant Bayer 205 level (above 1 mg./100 ml. of plasma) than does a single injection of a much larger amount. Since this problem is of considerable interest to those who are concerned with the prophylactic use of the drug in tropical countries, it was decided to extend these observations in order to obtain continuous curves to show the amount of Bayer 205 in the plasma of rabbits following (*a*) a large dose of the drug (equivalent to 6·3 g. for a 70 kg. man), and (*b*) three injections of one-third this amount, the three injections being made at intervals of about 5 weeks.

Experimental details. Three rabbits (group A) each received an intravenous injection of 0·09 g. Bayer 205/kg., and three others (group B) received 0·03 g./kg. Further injections, each of 0·03 g./kg., were given to the second group 36 days and 73 days after the first injection.

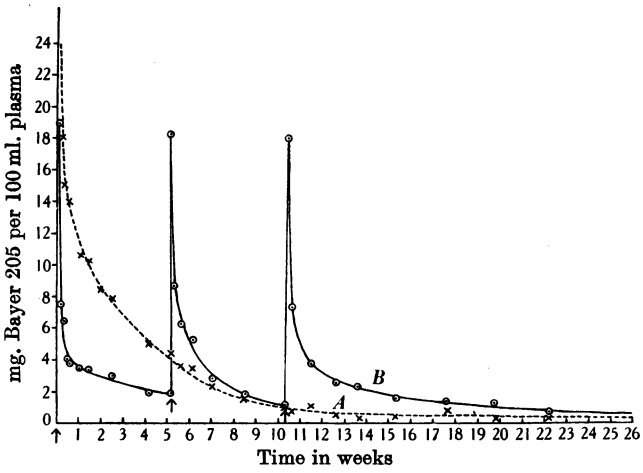

Fig. 1. Bayer 205 in the plasma of rabbits following the injection of the drug. ×---× Group A (one injection). o—o Group B (three injections). The arrows indicate when the injections were made.

Blood samples (about 6 ml.) were withdrawn from the marginal vein of the ear before, and 3 and 24 hr. after, each injection and at suitable intervals during the remaining periods. The blood was oxalated and centrifuged immediately. The amount of Bayer 205 in the plasma was then determined as previously

described [Dangerfield *et al.* 1938]. The determinations were made with a colorimeter and in most cases by the comparator method described in this paper; no significant difference between the two results was observed. Determinations of the "blank value" for normal rabbits were made at frequent intervals. These proved to be practically constant at the equivalent of approximately 1 mg. of Bayer 205/100 ml. of plasma and all values for the experimental animals have been corrected accordingly.

The results of this experiment are recorded in Fig. 1, the values recorded representing averages for the two groups of rabbits.

Discussion

Further study has shown that the previously described method of determination of Bayer 205 (or Antrypol) in plasma [Dangerfield *et al.* 1938] presents few difficulties and is satisfactory for the determination of amounts of the drug which are likely to be significant. No serious modification of the method has been found necessary, but the use of a Lovibond comparator with appropriate discs renders the determination much easier and makes it possible for measurements to be made when a colorimeter is not available. Throughout the whole of this work the authors have kept in mind the probability that workers in tropical countries, carrying out investigations under somewhat difficult conditions, might wish to make Bayer 205 determinations. For this reason, the observation made here, that serum and plasma give the same values, will be of special interest. Further experiments are now being made to determine the best method of preserving serum which will allow accurate determinations to be made several days or even weeks after the blood has been collected.

A considerable amount of time has been devoted to rendering the method applicable to urine, for such determinations will have to be made before a true picture of the behaviour of Bayer 205 in the body can be obtained. Although there is little or no chemical evidence that the drug is excreted in the urine after its injection into an animal, there is no doubt that this excretion does occur. The investigations on man now being carried out, involving an attempt to trace the fate of most of the Bayer 205 injected, necessitate measurement of the excretion, and the method described in this paper appears to be quite satisfactory for this purpose. The method is, unfortunately, more complicated than that for plasma or serum, but there is as yet no indication that urinary Bayer 205 determinations on patients receiving this drug will normally be needed. The more important value, as far as treatment is concerned, is almost certainly the plasma Bayer 205 level. If it is found necessary to make determinations on urine, the latter can be preserved by the addition of toluene, and the hydrolyses and determinations made at a later date.

The results of the experiment on rabbits confirm the suggestion made previously that for the maintenance of a significant level of Bayer 205 in the plasma it is better to have a series of injections rather than one massive dose (cf. Fig. 1). No evidence has yet been obtained that the latter builds up a higher reserve in the body, and from other evidence available it seems probable that a considerable part of the extra Bayer 205 injected in the large dose is excreted in the urine during the first few days. The amount of Bayer 205 or Antrypol usually injected into man for the curing or prevention of sleeping sickness varies very considerably [cf. Findlay, 1930, pp. 262–8], but the customary dose is 1 or 2 g. for the average man. These injections are often repeated at intervals of a few days or every week, and the total amount injected over a period of 6 months may be 5 g. or even as much as 7–10 g.

The long retention of Bayer 205 in the animal body is remarkable. It seems probable that the persistence of Bayer 205 in the plasma and tissues accounts for the very satisfactory prophylactic value of this drug as far as sleeping sickness is concerned. Information which would be very useful in this connexion can perhaps be classified as follows: (i) the minimum plasma–Bayer 205 level which will normally protect against sleeping sickness when the individual is exposed to infected tsetse flies, (ii) the best method of ensuring that this level will be exceeded without excessive administration of the drug, and (iii) the simplest way of making Bayer 205 determinations on the plasma of patients. The method described in this paper is suitable for the last-named determinations in ordinary field laboratories, but the other questions cannot be dealt with so readily. The minimum "protective" Bayer 205 level for man can best be established by numerous determinations on patients who develop the disease after treatment with the drug. In that way it may be possible to say that if the amount of Bayer 205 in the plasma falls below a certain value the protection against trypanosomiasis is not adequate. Another method, and the one which the authors propose to adopt, involves experiments on small animals (rabbits, mice etc.), since the natural conditions (man as the experimental animal and infection by the bite of infected tsetse) cannot be reproduced readily in this country.

The best method of maintaining a protective level of Bayer 205 in the plasma can only be satisfactorily established when the average minimum protective level has been fixed, but for present purposes it might be assumed (very tentatively) that values above 1 or 1·5 mg./100 ml. of plasma may be significant under normal conditions. If this is not so, the protective effect observed in investigations on volunteers injected with Bayer 205 and subsequently exposed to the bites of infected tsetse flies [Duke, 1934] and the almost universal agreement that small amounts of the drug will give protection for several weeks [cf. review of literature by Findlay, 1930] are difficult to explain; otherwise it would be necessary to assume that the protective action is not directly related to the Bayer 205 present in the plasma and in tissue fluid. In the light of these chemical studies, therefore, there does not appear to be justification for either a very large dose of the drug (greater than 2 g. for an average man) or for several injections at very short intervals (e.g. every few days). A series of three injections made every 4 or 5 weeks should serve to maintain the plasma-Bayer 205 level above 1 mg./100 ml. for a total period of 4 or 5 months. It is not suggested however that the valuable clinical observations of those who prefer to give several injections over a period of 1 or 2 weeks should be disregarded, for until a "minimum protective level" (if such a value does exist) can be determined, it would be undesirable to contrast critically the various methods of prophylactic treatment.

The possibility that Bayer 205 is stored in certain parts of the body is being studied at the present time. These experiments are as yet incomplete, but there seems to be no doubt that the drug is not stored to any significant extent in organs such as the liver, spleen etc. Other experiments, which will be described in a later paper, show that some type of combination occurs between Bayer 205 and plasma proteins, and the long retention of the drug in the animal body is most probably due to its combination with plasma and tissue proteins.

Summary

1. Further observations have been made on the method of determination of Bayer 205 (or Antrypol) previously described [Dangerfield *et al.* 1938]. The technique has been simplified by the preparation of coloured discs for a Lovibond comparator.

2. Plasma and serum obtained from a Bayer 205-containing blood contain the same amount of the drug.

3. A modification of the method has been devised for the determination of small amounts of Bayer 205 in urine. The difference between the values for the urine and an ultrafiltrate obtained by filtration through a standard collodion membrane is a measure of the Bayer 205 present. This difference gives a minimum value which can be rendered more accurate by the use of a correction value. This factor is usually between 1·10 and 1·25, and it can be determined quite easily.

4. Investigations have been carried out which support the suggestion made in a previous paper that a course of injections of the drug results in a more satisfactory maintenance of a significant amount of Bayer 205 (or Antrypol) in the plasma over a long period than does a single injection of the same total amount.

5. Some possible reasons for the long retention of the drug in the plasma are tentatively discussed.

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