XI. STUDIES ON BAYER 205 (GERMANIN) AND ANTRYPOL

I. THE DETERMINATION OF SMALL AMOUNTS OF BAYER 205 (AND ANTRYPOL)

II. THE PERSISTENCE OF BAYER 205 IN THE BLOOD STREAM AFTER INJECTION INTO ANIMALS

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BAYER 205 and Antrypol, besides having very high curative effects in trypanosomiasis, have been shown to possess marked prophylactic or preventive properties as far as this disease is concerned. Although the *in vivo* trypanocidal action of these drugs has not yet been fully explained, it seems probable that their prolonged prophylactic action is due to the fact that they are retained in the blood and probably in the tissues for some considerable time after injection. It seems desirable, therefore, that more information should be obtained about the "metabolism" of these drugs in the body. At a conference on Tsetse and Trypanosomiasis (Animal and Human) Research held at Entebbe a few years ago [Report, 1934] the view was expressed "that it was important that the question of the rapidity of excretion of Bayer 205 from the body should be investigated" and "it was recommended that the Medical Research Council should be approached with a request to work on this problem". The Medical Research Council having taken up the matter accordingly, it was at their request that the experiments described in this paper were made.

The first essential was an accurate method for the determination of Bayer 205 in plasma, and if possible, in other body fluids, and after much search, the method described in the first part of this paper was devised. This method gives satisfactory results with plasma, but considerable difficulties were encountered when attempts were made to apply it to urine. Various modifications for the urine determinations have been tried and although some are quite promising, it is not possible as yet to place any great reliance on the results obtained. The present report will therefore be confined to the problem of the retention of Bayer 205, and the mode of elimination will be left to a future date. These early investigations have been made on small animals (rabbits and dogs), but similar experiments are being carried out with man as the experimental animal. At the same time efforts are being made to determine the minimum plasma Bayer 205 level which will prevent infection when various strains of trypanosomes are injected into small animals.

I. THE DETERMINATION OF SMALL AMOUNTS OF BAYER 205 (AND ANTRYPOL)

Two methods for the determination of Bayer 205 in body fluids have been described. Steppuhn and Utkina-Ljubowzewa [1924] coupled the products obtained by alkaline hydrolysis of Bayer 205 with *p*-diazobenzenesulphonic acid in acetic acid solution, and compared the orange colour obtained with standards. This method has been criticized by Lang [1931] on the grounds that loss of the material to be determined occurs during alkaline hydrolysis and further that the diazo-compound couples with substances normally present in the blood to give coloured compounds. The present authors have confirmed these findings of Lang and have additional criticisms of the method to offer. When determinations are made on blood serum or plasma, protein precipitates are produced which carry down a certain amount of the orange colour which is ultimately produced. In the case of determinations on urine, it has been found that normal urines invariably give a very marked coloration when the diazo-compound is added. In view of these facts it is concluded that this method is not suitable for the detection or determination of Bayer 205 in body fluids.

Attention was then turned to the method of Lang [1931] in which the aromatic amines liberated by the acid hydrolysis of Bayer 205 (*m*-aminobenzoic acid, *m*-amino-*p*-toluic acid and α -naphthylamine-4:6:8-trisulphonic acid) are diazotized and coupled, in acetic acid solution, with α -naphthol. The orange-yellow colour produced is estimated colorimetrically by comparison with a similarly treated standard solution of Bayer 205. Although the method is distinctly better than that of Steppuhn and Utkina-Ljubowzewa, it is not completely satisfactory. The acid hydrolysis employed in this technique leads to the production of brown or black pigments from urine and from blood plasma or serum. The pigments are partially removed by treatment with kaolin and filtration, but the "urine" filtrates have a yellow colour which is similar to that ultimately produced when the α -naphthol is coupled with the diazotized hydrolysed Bayer 205. The filtrates from plasma and serum also contain pigments which are not completely removed by kaolin and which interfere with the subsequent matching of the orange-yellow colours.

Attempts were made to overcome these difficulties by the use of coupling agents other than α -naphthol in the hope of producing deeper colours such as reds or blues. Tests were made with a large number of *N*-substituted naphthylamines and other compounds used for coupling with diazo-compounds, and ultimately it was found that methyl- α -naphthylamine gave a magenta colour when coupled in acetic acid solution with the diazotized products of the acid hydrolysis of Bayer 205. Subsequent experiments showed that the coupling was quantitative, and further, that the slight brownish yellow colour of hydrolysed and kaolin-treated plasma interfered to a very slight extent only with the matching of the colours. The method for the estimation of small amounts of Bayer 205 and of Antrypol, described in this paper is, therefore, a modification of the Lang technique. The modifications have been introduced in order to obtain the optimum conditions for the production and stabilization of the magenta colour obtained when methyl- α -naphthylamine is employed as coupling agent.

Description of the method

The principle of the method consists in hydrolysing Bayer 205 (or Antrypol) with conc. HCl; the aromatic amines produced are diazotized, and after removal of excess of HCl with sodium acetate the diazo-compounds are coupled with monomethyl- α -naphthylamine. The magenta colour produced is compared with that obtained from a similarly treated standard solution of Bayer 205 or Antrypol. The methyl- α -naphthylamine was prepared according to Fischer [1895]. It can also be purchased from Messrs May and Baker, Ltd., Dagenham.

The following reagents are required:

- (1) Concentrated HCl.
- (2) Kaolin (Society of Leather Trades Chemists' specification).
- (3) 0.5 % sodium nitrite.
- (4) Saturated urea solution.
- (5) 30 % sodium acetate.
- (6) 0.5 ml. methyl- α -naphthylamine (or 0.2 g. of the hydrochloride) in 100 ml. of 50 % acetic acid (% by volume).

To 1–3 ml. of the oxalated plasma in a 10 ml. graduated tube, are added 3 ml. of conc. HCl. A glass bulb is placed in the top of the tube, which is then heated in the boiling water bath for 6 hr. The cooled brown or brownish black mixture is diluted with distilled water to 10 ml., treated with approximately 3 g. of previously heated kaolin, shaken well and filtered. 2 ml. of the filtrate are treated with 1 drop of 0.5 % NaNO_2 , followed after 1 min. by 2 drops of saturated urea solution, the mixture being well shaken after each addition. After 2 min., 3 ml. of 30 % sodium acetate solution are added, followed immediately by 1 ml. of the methyl- α -naphthylamine solution. The colour develops rapidly and after 10–15 min. is matched colorimetrically against a standard.

The standard solutions are prepared as follows. A solution of Bayer 205 (e.g. 100 mg./100 ml.) is treated with an equal volume of conc. HCl and heated for 6 hr. in the boiling water bath. From this solution, which appears to be stable for a considerable time, various standards are made by dilution with 3*N* HCl, the most useful being those containing 0.25, 0.5, 0.75, 1.0, 1.5, 2.0 and 3.0 mg. Bayer 205 per 100 ml. 2 ml. of each standard are diazotized, treated with urea and coupled with methyl- α -naphthylamine as described above. If the solution under examination gives a deeper colour than that produced by the 3 mg./100 ml. standard, less of the filtrate is used, 3*N* HCl being added to give a volume of 2 ml. before diazotization. Solutions giving a less intense colour than that produced by the 0.5 mg./100 ml. standard, or solutions containing so much yellow colour that matching in the colorimeter is difficult, are matched in a comparator. The solution used for compensation in these cases consists of the filtrate treated with NaNO_2 , urea, sodium acetate and 1 ml. of 50 % acetic acid.

The concentration of Bayer 205 in the biological fluid (in mg. per 100 ml.) is given by the following formula:

$$S = \frac{10}{v_1} \times \frac{2}{v_2} \times \frac{a}{b} \times S,$$

where v_1 = volume of fluid hydrolysed (plasma, serum etc.); v_2 = volume of filtrate diazotized; a = colorimeter reading of standard; b = colorimeter reading of solution; S = concentration of standard (mg. Bayer 205 per 100 ml.).

The following points have been found to be important.

- (1) The correct balance between HCl and sodium acetate must be main-

tained. Any dilution of the "kaolin-filtrate" should be made with 3*N* HCl, and 2 ml. of this diluted solution used for the preparation of the red colour.

(2) No excess of nitrous acid should remain in the solution after the treatment with urea. The contents of the tube should be well shaken to make certain that no nitrous acid remains on the side of the tube.

(3) It is important that the prescribed time should be allowed for diazotization and for the destruction of excess nitrous acid. Most satisfactory results are obtained when these processes are accurately timed.

(4) The methylnaphthylamine reagent should be added immediately after the sodium acetate.

(5) Normal sera usually give a small blank value, presumably due to amines present in the plasma or produced by the hydrolysis. This blank value for rabbits appears to vary slightly from time to time, but the cause of this variation has not yet been established. In these investigations determinations have been made on control animals and the values given in this paper have, where necessary, been corrected for the small blank (usually equivalent to 0.8 or 0.9 mg./100 ml. of Bayer 205).

When attention is paid to these points the method is relatively simple and gives satisfactory results when duplicates are compared and when determinations are made on sera or plasmas containing known amounts of Bayer 205 (cf. Table I).

Table I. *Determination of Bayer 205 (and Antrypol) in water, plasma and serum*

Medium	Amount of drug added mg./100 ml.	Amount recovered mg./100 ml.
Water	10.0 Bayer 205	9.9
	5.0 "	5.1
	2.0 "	2.0
	10.0 Antrypol	10.0
	5.0 "	5.0
Plasma (rabbit)	5.0 Bayer 205	5.1
	2.0 "	2.2
Serum (horse)	11.4 Antrypol	11.3
	5.7 "	5.6
	2.3 "	2.4
	5.7 Bayer 205	5.9
	2.3 "	2.4

Methyl- α -naphthylamine undergoes some decomposition, especially when exposed to light, and becomes red-brown. This colour is not usually sufficient to necessitate correction of the results, but in more recent work it has been found desirable to purify the reagent by conversion into the hydrochloride. Experiments so far carried out show that the solid hydrochloride undergoes no apparent decomposition when kept in the dark for several weeks. It is also more economical to use, for the 0.5 % solution of the base can be replaced by a solution containing 0.2 g. of the hydrochloride in 100 ml. of 50 % acetic acid (by volume). Where neither the base nor the hydrochloride is available, recrystallized α -naphthylamine can be used but the colour with this reagent is not quite as bright a red, does not develop as quickly, and is more difficult to match with the standards than is the colour with methyl- α -naphthylamine.

Very recently efforts have been made (in collaboration with Dr F. Hawking) to utilize a Lovibond comparator for this determination, and coloured discs are

being made by "The Tintometer Ltd." When this object has been achieved it is hoped that determinations of Bayer 205 will be possible in small field laboratories in tropical countries where a colorimeter is not available.

DISCUSSION

(a) *The hydrolysis of Bayer 205 and Antrypol.* In agreement with Lang [1931] it has been found that if a mixture of equal volumes of plasma and conc. HCl is heated in a boiling water bath, hydrolysis of any Bayer 205 present is complete in 5–6 hr. (cf. Table II). Heating for 3 or 4 hr. longer causes very little loss of the amines produced by the hydrolysis. Almost identical results, both as regards the amount present in solution and the rate of hydrolysis by HCl, have been obtained with Antrypol (B.D.H.). In all the experiments carried out with this colorimetric determination, Antrypol has behaved exactly like Bayer 205, and for these determinations standards prepared from either of the hydrolysed drugs can be used. In view of the considerable difference in the cost of these two preparations it is proposed to carry out most of the later experiments with Antrypol.

Table II. *Rates of hydrolysis of Bayer 205 and Antrypol. % of Bayer 205 or Antrypol hydrolysed*

Bayer 205	57.1	79.1	90.7	95.5	98.1	100.0	100.0	99.1
Antrypol	54.1	79.5	89.9	95.1	99.3	99.7	100.0	98.4
Time (hr.)	1	2	3	4	5	6	8	10

Experimental details

Solutions of Bayer 205 and Antrypol containing 10 mg./100 ml. were treated with an equal volume of conc. HCl. Twenty 6 ml. samples of each solution were placed in a boiling water bath and two tubes of each set were removed after various intervals. The amount of hydrolysed Bayer 205 in each tube was determined and the average figure for each pair of duplicates was used for the calculation of the percentages given in the table. Solutions of hydrolysed Bayer 205 were used as standards for these determinations.

(b) *Decoloration.* Lang [1931] states that kaolin removes the dark-coloured pigments present in hydrolysed plasma without loss of the products of hydrolysis of Bayer 205. This observation has received general confirmation in the present investigations, but it must be recorded that with solutions containing small amounts of Bayer 205 (less than 2 mg./100 ml.) treatment with kaolin results in a small but detectable loss (equivalent to about 0.15 mg./100 ml. of Bayer 205). Where necessary, allowance should be made for this adsorption. No adjustment is needed with plasma determinations, since there is a corresponding loss in the blank.

Decoloration with kaolin is not complete, particularly in the case of urine estimations, and search has been made for some more satisfactory method. Other adsorbents have been found to be unsatisfactory, for removal of the pigments is usually accompanied by a loss of Bayer 205. Heated kaolin is, however, a little more effective than the unheated material, and in all the later experiments the kaolin has been heated moderately strongly in a boiling tube for about 1 min. before addition to the diluted hydrolysed plasma.

(c) *Diazotization and coupling.* In the early stages of the experiments it was found that the colour produced by coupling the diazotized products of acid hydrolysis of Bayer 205 with methyl- α -naphthylamine was slowly destroyed, and this instability was traced to the presence of an excess of nitrous acid. The

addition of urea before neutralization with sodium acetate removes this excess of nitrous acid and the colour then remains constant for several hours.

It was also found that whilst the presence of excess of HCl diminishes both the depth of the colour and the rapidity with which it is produced, excess of sodium acetate, which aids both these factors, makes the solution turbid owing to the precipitation of methyl- α -naphthylamine. It is necessary, therefore, to control the amounts of HCl and sodium acetate. Conditions were ultimately chosen which gave the most rapid production of a maximal colour which would remain clear for at least 2 hr. Thus the 2 ml. sample used for analysis must be approximately 3*N* in HCl and the sodium acetate added after diazotization must be 30 %. If turbidity is produced in individual solutions, they must be cleared by centrifuging, but this is rarely necessary.

It is also important that the methyl- α -naphthylamine solution should be added immediately after the sodium acetate. Any appreciable delay at this stage causes loss of the diazo-compounds, probably owing to their coupling with compounds present in the hydrolysed plasma. As the solution of methyl- α -naphthylamine becomes darker on standing, it is necessary to make up this reagent every 3–4 days.

(*d*) *Accuracy of the method.* The method has proved satisfactory for the determination of small amounts of Bayer 205 and Antrypol, and it gives fairly accurate results with even the smallest amounts of these drugs which have been shown to exercise a definite trypanocidal effect. With aqueous solutions as little as 0.2 mg./100 ml. of Bayer 205 can readily be detected, and accurate determinations made when the concentration exceeds 0.6 mg./100 ml. In the case of plasma, where an allowance for the blank value must be made, it is possible to detect 0.4 mg./100 ml. and to determine with sufficient accuracy amounts greater than 0.6 mg./100 ml. When the concentration in the plasma exceeds 2 mg./100 ml., the error is not greater than that normally associated with the matching of colours in a colorimeter (cf. Table I).

Up to the present it has not been found possible to make accurate determinations of Bayer 205 in urine, for the blank values obtained for normal urines, with this or any similar method, are considerable and are not sufficiently constant to warrant any conclusion being reached as to the amount of Bayer 205 present. Amines and possibly other diazotizable substances are normally found in the urine to such an extent and with such wide variation in the amounts (equivalent to 2–5 mg./100 ml. of Bayer 205), that the method described here, and still more those of Steppuhn and Utkina-Ljubowzewa [1924] and Lang [1931], can only be used to give an approximate value for Bayer 205 in urine. Attempts have been made to remove the diazotizable substances from the urine before hydrolysis, but the results are not yet sufficiently satisfactory to justify the publication of this modification.

It should be pointed out that this method, and any other method involving the same principles, can only give a figure for the amount of Bayer 205 plus related substances present in the plasma. It does not appear likely that there will be any appreciable amount of simple amines in the blood plasma following the injection of Bayer 205, but there is still the possibility that this drug is partially degraded or converted into other derivatives which remain in the blood stream. These Bayer 205 derivatives would probably be hydrolysed by HCl to give amines which would give red colours when diazotized and coupled with methyl- α -naphthylamine. There is evidence, however, that after its injection part of the Bayer 205 is retained in the body in the form of the unchanged compound. In the experiments recorded in the second part of this paper the

assumption is made that the results obtained are a measure of the amount of unaltered Bayer 205 in the plasma, but further work is proceeding to determine, if possible, whether there is any degradation of this drug in the animal body.

II. THE PERSISTENCE OF BAYER 205 IN THE BLOOD STREAM AFTER INJECTION INTO ANIMALS

It has long been recognized that the injection of Bayer 205 into animals infected with trypanosomes not only causes disappearance of the trypanosomes from the blood but also confers on the animals an immunity towards this type of infection. This immunity lasts for a varying length of time, depending on the amount of drug injected, the nature of the animal and the infection, and possibly on a variety of other factors. The prophylactic action of Bayer 205 has been demonstrated with various small animals (the rat, guinea-pig and monkey) and with man, and the immunity produced is effective against the bites of infected tsetse flies or the injection of trypanosomes into the animal. (For a survey of the literature on this subject the review of Findlay [1930], and the papers of Mayer [1928], Browning & Gulbransen [1934] and Duke [1934] should be consulted.)

The fact that a single injection of the drug into a monkey, an animal which is very sensitive to *T. rhodesiense* and *T. brucei*, will give protection for at least one month against infection from the bite of tsetse infected with these trypanosomes, "places Bayer 205 in a class by itself as a prophylactic of unique properties and immense possibilities" [Duke 1934]. Certain drugs can be used to give protection against other diseases, but there appear to be few instances where the prophylactic effect lasts for more than a few days. One exception to this general rule is the remarkable protection, against *T. brucei* infection, conferred by one subcutaneous injection of "Styryl-245" [Browning & Gulbransen, 1934]. This protection may last up to almost one year, and the prolonged effect is attributed to the formation of a local deposit from which the active substance passes very slowly into the blood stream. A similar explanation could not, of course, be offered to explain the long protective action of Bayer 205. Little is known about the length of time that this drug remains in the body after injection, or about the chemical explanation for its retention. The trypanocidal effect of Bayer 205 may be due to a direct action on the trypanosomes or an indirect mechanism, but whatever the reason, it is important that more should be known about the "metabolism" of this drug in the animal body.

EXPERIMENTAL

The determinations of Bayer 205 or Antrypol in the plasma of rabbits and dogs were carried out by the method described in Part I of this paper. Samples of blood (6-10 ml.), removed from the marginal vein of the ear, were oxalated and then centrifuged almost immediately. Determinations were occasionally made on duplicate samples of plasma, but generally only single samples were used. In these latter cases duplicate determinations were made on the filtrate produced after hydrolysis and kaolin treatment of the plasma. For injection, the Bayer 205 and Antrypol were dissolved in 0.9% NaCl and the solutions sterilized by boiling. The injections were all made intravenously.

Table III. *Amount of Bayer 205 (mg./100 ml.) in the blood of rabbits after a single injection of 0.1 g. per kg.*

Rabbit No. 468	201	142	—	73	48	33	30	21	14.5
Rabbit No. 449	204	—	86	—	51	32	25	18	11
Time after injection	5 min.	30 min.	1 hr.	3 hr.	6 hr.	24 hr.	30 hr.	48 hr.	4 days
Rabbit No. 468		13.5	10.5	rabbit died					
Rabbit No. 449		10	6.2	2.6	2.5	2.0	1.2	0.5	
Time after injection		6 days	11 days	6 wks.	14 wks.	19 wks.	23 wks.	30 wks.	

Table IV. *Bayer 205 in the plasma of rabbits following injection of the drug (in mg. per 100 ml.)*

Group A. One large injection					Group B. Five small injections					
Time after injection	No. 486	No. 483	No. 484	Av.	Time after first injection	Time after previous injection	No. 480	No. 481	No. 482	Av.
→					→					
2½ hr.	88	94	85	89	2½ hr.	2½ hr.	29.3	33	29.0	30.4
4 days	24.7	20.6	25.7	23.7	4 days	4 days	2.7	5.2	3.0	3.6
→					→					
8 „	14.0	14.5	15.7	14.7	8 days	20 hr.	9.0	16.0	11.0	12.0
14 „	9.0	11.0	12.0	10.7	14 days	7 days	3.0	7.0	3.8	4.6
→					→					
21 „	5.0	7.3	7.0	6.4	21 „	7 „	7.1	6.7	5.7	6.5
→					→					
32 „	4.0	6.3	5.3	5.2	32 „	11 „	6.6	6.5	5.2	6.1
→					→					
39 „	3.1	4.4	3.7	3.7	39 „	7 „	8.4	9.4	8.4	8.7
53 „	3.2	4.2	3.5	3.6	53 „	21 „	6.4	6.4	6.6	6.5
67 „	2.0	3.0	2.0	2.3	67 „	35 „	4.5	3.1	4.5	4.0
90 „	1.7	1.8	1.6	1.7	90 „	58 „	2.4	1.4	2.9	2.2
17 weeks	1.0	1.5	1.1	1.2	17 weeks	12 weeks	1.8	1.5	2.1	1.8
25 „	0.8	1.1	1.0	1.0	25 „	20 „	1.1	1.0	1.2	1.1

Group A received one injection of 0.14 g. Bayer 205 per kg.
Group B received five injections of 0.028 g. Bayer 205 per kg.
The arrows in the above table indicate when the injections were made.

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

In a preliminary experiment two rabbits were injected with 0.1 g. Bayer 205 per kg., a dose which is, of course, considerably greater than that normally given to man. Blood samples were removed before the injection and after 5 min., 30 min., 1, 3, 6 and 24 hr., and later at fairly regular intervals. One of the rabbits died (as a result of an unrelated infection) 11 days after the injection, but the other was studied for a total period of 7 months. The results in Table III, show that 6 or 7 months after the injection there is still a determinable and significant amount of the drug in the blood plasma.

In a second experiment two groups each of 3 rabbits were taken. Each rabbit in the first group (group A) received a single dose of 0.14 g. Bayer 205 per kg. The rabbits of group B received over a period of 32 days five injections, each of 0.028 g. Bayer 205 per kg. (equivalent to 2 g. for a 70 kg. man). Determinations of the plasma Bayer 205 were made for 6 months after the first injections. The results of these experiments (Table IV and Fig. 1) indicate that, with a series of injections of small amounts of the drug, the amount present in the plasma is greater, over the major portion of the experiment, than it is when the same total amount of Bayer 205 is injected in one dose.

In another experiment, Antrypol (B.D.H.) was injected into a dog, with a view to finding out whether this preparation behaved exactly like Bayer 205, and partly with the object of measuring the rate of its excretion in the urine. The dog received an intravenous injection of 0.03 g. per kg. and a similar dose 37 days later. Blood samples were taken before the first injection and for a total period of 14 weeks, and the whole of the urine voided during the experiment was collected. For reasons given earlier in this paper, no reliance can be placed on the actual figures for urinary Bayer 205, but with this reservation, it can be stated that they indicate that fairly appreciable amounts of the drug are eliminated in the urine during the first few days. After this initial period, the amount present in the urine is so small that it is less than the variation in the blank values (the "Bayer 205" values for normal urine). The results obtained with the blood samples (cf. Table V) show that Antrypol behaves like Bayer 205, and that two injections of a small amount are sufficient to maintain a significant amount of the drug in the plasma for a period of three months.

Table V. *Amount of Antrypol (determined as Bayer 205) in the plasma of a dog following two intravenous injections of the drug*

Weight of dog: 4.5 kg.

Injections: 0.13 g. Antrypol at the start and 0.13 g. after 38 days.

Time after injection	17 days	37 days	53 days	14 weeks
Antrypol in plasma (mg. per 100 ml.)	2.7	0.8	1.2	1.0

DISCUSSION

For prophylactic purposes it is obviously desirable that the blood Bayer 205 level should remain above a certain, but at present unknown, minimum value. With the method of determination of this drug described in this paper it is hoped that it will be possible in the future to obtain some figure, even if it is only approximate, for the minimum *in vivo* trypanocidal concentration for various species of trypanosomes.

After the method of determination of the drug had been established, the first experiment of the present investigation was designed to give a general idea of the length of time that injected Bayer 205 remains in the body. In this but no other experiment, the dose of Bayer 205 was distinctly higher than the

equivalent of a normal single dose for man. The plasma Bayer 205 level of the injected animals fell very rapidly during the first 6 hr. and thereafter the fall was less rapid. 19 weeks after the injection the plasma contained Bayer 205 to the extent of 2 mg./100 ml. and after 23 weeks 1.2 mg./100 ml. (Table III). As will be mentioned below, it is possible that even in these low concentrations the drug may have trypanocidal effects.

Other experiments were concerned with a comparison of the effects produced by a single large injection with those of a series of smaller doses. The results given in Table IV and Fig. 1 are in favour of the series of injections, and the differences are certainly significant in the important part of the experiment where the plasma Bayer 205 level has fallen to fairly low values; e.g. in the period 7–17 weeks after the first injection. Experiments are now being carried out on man and on rabbits to attempt to establish this superiority of a series of injections. Partly on theoretical grounds and partly on the results of these investigations, it might tentatively be suggested that the best results might be obtained by injecting a fairly high initial dose with smaller doses (e.g. 1 g. Bayer 205 to a 70 kg. man) one week later and subsequently at longer intervals. After each injection there is rapid excretion of a considerable amount of the injected drug, but every subsequent injection, if the delay is not too long, appears to build up a higher “persistent” plasma Bayer 205 level. In this connection it is of interest to note that Duke [1934], as a result of his work with human volunteers and with monkeys exposed to the bites of infected tsetse flies, suggests that the administration of a second dose 3 weeks or so after the first injection enhances the protective effect of Bayer 205.

These investigations have not yet been concerned with the nature of the trypanocidal action of Bayer 205. There still appears to be some doubt as to how this drug causes the death of trypanosomes when it is injected into an infected animal. The *in vitro* trypanocidal action does not seem sufficiently high to account for its powerful *in vivo* action, and it has been suggested that it has an opsonin-like effect on the trypanosomes [Reiner & Köveskuty, 1927; Jancsó & Jancsó, 1934, 2]. The concentration of Bayer 205 needed to kill trypanosomes *in vitro* within 24 hr. at 37° is about 62 mg./100 ml. [Yorke *et al.* 1931]. Jancsó & Jancsó [1934, 1] report, however, that a concentration of 1.7 mg./100 ml. is sufficiently strong to kill trypanosomes and that a trypanocidal action is exerted in concentrations as low as 1.25 mg./100 ml. If the latter figures are accepted, the results recorded in the present paper would suggest that a series of five injections of a “normal” dose of Bayer 205 may give protection for a period of at least 5 months or about 4 months after the last injection. It should be pointed out, however, that Hawking [1937], working with *T. rhodesiense*, has recently reported that an *in vitro* concentration of 125 mg./100 ml. of Bayer 205 is required to kill 100% of the trypanosomes although the majority of the organisms may be killed by 31 mg./100 ml.

Strong experimental support for the “opsonic theory” is offered by these investigations of Hawking [1937], for it has been shown that trypanosomes which have been exposed for more than 2 hr. to a concentration of 10 mg./100 ml., a concentration considerably below that required to kill the trypanosomes, are incapable of infecting mice. Exposure of the trypanosomes to 1 mg./100 ml. of Bayer 205 is occasionally but not usually sufficient to cause loss of infective power. From these results, and when the relatively weak *in vitro* trypanocidal power of Bayer 205 is considered, it seems probable that this drug destroys trypanosomes in the animal body in part at least by an indirect action, possibly by a sensitizing mechanism. Whether the special prophylactic

properties of Bayer 205 are due to its direct action on the trypanosomes or to some indirect action, it must be remembered that the period of exposure of the organisms to the drug is very much longer in the body than it is in *in vitro* experiments. Where a low concentration of the drug is found *in vitro* to possess trypanocidal or harming effects, it is not improbable that even lower concentrations will be effective in the body.

One of the most interesting results of the present investigations is the length of time that Bayer 205 remains in the body. This retention of the injected drug has been noted previously by several authors [for the literature see Findlay, 1930], but in most cases no real estimate of the amount present in the blood could be given and the duration of the experiment was usually comparatively short. An appreciable amount of Bayer 205 is excreted in the urine during the first week or so after the injection but the remainder is excreted very slowly. Several possible explanations might be offered to account for this retention; for example, there might be storage in the liver or some other organ (although Lang [1931] states that there is no removal by the liver), or there might be combination with proteins or some other constituents of the blood. The inhibitory effect on blood coagulation and the anti-complementary power of Bayer 205 suggest that it is capable of combining with certain blood proteins or protein complexes, but further speculation about the cause of the retention of the drug in the body will be left to a subsequent publication.

SUMMARY

1. A method, based on that introduced by Lang [1931], is described for the determination of Bayer 205. The Bayer 205 is hydrolysed by strong HCl and the amines produced are diazotized and coupled with methyl- α -naphthylamine. The red colour produced is compared colorimetrically with those obtained from standard solutions of hydrolysed Bayer 205.

2. Amounts of Bayer 205 as low as 0.2 mg./100 ml. in water (or a quantity of 0.004 mg.) can readily be detected, and accurate determinations made when the concentration exceeds 0.6 mg./100 ml. With blood plasma, amounts greater than 0.6 mg./100 ml. can be determined with sufficient accuracy. Owing to a high and variable blank value, it has not yet been possible to adopt this method for the determination of Bayer 205 in urine.

3. Antrypol (B.D.H.) behaves exactly like Bayer 205 when hydrolysed and determined by this method. The rates of hydrolysis of these two preparations by HCl are practically identical.

4. After intravenous injection of Bayer 205 into rabbits and dogs, the amount of the drug in the plasma falls rapidly during the first few days, owing in part to excretion in the urine, but after this initial period the diminution is less rapid.

5. Five or six months after the intravenous injection into a rabbit of a relatively large dose of Bayer 205 (0.1 g. per kg.) there is still a significant amount of the drug in the plasma (at least 1 mg./100 ml.). Two injections, each of 0.03 g. per kg., into a dog resulted in a plasma Bayer 205 level of 0.8 mg. or more per 100 ml. for 14 weeks after the first injection.

6. Preliminary experiments have shown that rabbits which have received several smaller doses of Bayer 205 over a period of a few weeks, have more Bayer 205 in their plasma in the later stages of the experiment than have rabbits which received one injection of the same total amount. In these experiments, five injections each of 0.028 g. per kg., maintained the average level of Bayer 205 above 4 mg./100 ml. of plasma for about 9 weeks after the first injection, and after 20 weeks the level was still above 1 mg./100 ml.

7. The significance of these small amounts of Bayer 205 in the plasma is discussed in relation to the figures given by other authors for the minimum *in vitro* trypanocidal concentration of this drug. In view of the recent finding of Hawking [1937], that concentrations of 10 mg./100 ml. and sometimes as low as 1 mg./100 ml. destroy the power of trypanosomes to infect mice, it is suggested that the amount of Bayer 205 found in the plasma of an animal several months after a course of injections may be sufficient to account for the marked prophylactic action of this drug.

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