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PHARMACOLOGICALLY ACTIVE PEPTIDES IN THE BLOOD AND URINE OF ANIMALS INFECTED WITH *BABESIA RODHAINI* AND OTHER PATHOGENIC ORGANISMS

BY

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The blood and urine of mice and rats infected with *Babesia rodhaini* contain substances which stimulate the isolated guinea-pig ileum and rat duodenum. The amount of active material excreted increases as the infection increases. The active substances are stable to boiling with hydrochloric acid but not with alkali; they pass through a cellophane membrane and are soluble in hot ethanol. They are destroyed rapidly by papain and less rapidly by chymotrypsin, but are unaffected by trypsin or pepsin. Their action on smooth muscle is not affected by atropine, eserine, antihistamines, iproniazid, bretylium or by lysergic acid diethylamide. The active substances are probably peptides and there is evidence that the urine contains a mixture of peptides, some of which relax and some of which contract the rat duodenum. Similar active peptides appear in the urine of mice infected with *Plasmodium berghei*, *Trypanosoma rhodesiense*, *Streptococcus pyogenes* and Rift Valley fever virus.

Maegraith, Gilles and Devakul (1957) made a study of the pathological processes in puppies and dogs infected with *Babesia canis*. They showed that in severe and fatal cases the clinical progress bore " little direct relation to the prevailing degree of parasitic infection of the erythrocytes," and that the plasma often became lytic for both parasitized and normal erythrocytes. After severe lysis, the erythrocyte count, haemoglobin and packed cell volume often remained within normal limits, although the animal passed into a state resembling oligaemic shock. An injection of noradrenaline caused immediate, though temporary, recovery from this condition; a similar dramatic effect was reported by Maegraith, Devakul and Leithead (1956) in monkeys infected with *Plasmodium knowlesi*.

Maegraith *et al.* (1957) concluded that the pathological processes are non-specific and are common to other acute medical states, and that " the initiating factors may be physiologically active soluble substances of relatively simple nature derived from the parasite or as a consequence of host-parasite reaction or arising from tissue damage."

Severe or fatal shock also occurs in cattle infected with piroplasms, and may be brought about by treatment with effective drugs (Stephan and Esquibel, 1929). A similar phenomenon is sometimes observed in mice infected with *B. rodhaini* (Beveridge, 1953). It has been suggested that the destruction of parasites by the drug liberates substances which are toxic to the host. The liberation of " toxins " from protozoal parasites has often been suggested to explain physiological disturbances in the host, but pharmacological studies of the nature of these " toxins " have been few.

The present paper describes a preliminary study of the active substances present in the blood and urine of animals infected with *B. rodhaini* and other pathogenic organisms. Pharmacologically active peptides are shown to be present.

METHODS

Infections

Babesia rodhaini.—The strain used was the rat-adapted strain described by Beveridge (1953). Mice (20 g.) or rats (50 g.) were inoculated intraperitoneally with 0.1 ml. of infected rat blood containing 100 to 600m. parasitized red cells. The strain has increased in virulence since 1953 and now kills all mice in about 5 days. Rats did not die; maximal parasitaemia was reached in 5 to 6 days and then declined. In most experiments the parasites were counted daily in stained blood smears.

Plasmodium berghei.—The strain was obtained from the London School of Hygiene in 1949 and maintained in mice by blood passage. Mice were

infected by the intraperitoneal injection of 0.1 ml. of infected blood containing about 500 million parasitized red cells. The infection reached its maximum in the blood in 8 days; most of the mice died 6 to 8 days after inoculation.

Trypanosoma rhodesiense.—The strain (C) was isolated in Entebbe in 1939 and maintained in mice by blood passage. Mice were infected by the intraperitoneal injection of 0.5 ml. of diluted infected blood containing about 500,000 trypanosomes; the mice died 4 days after inoculation.

Streptococcus pyogenes.—A culture of a freshly isolated strain was obtained from University College Hospital. Mice were inoculated intraperitoneally with 0.05 ml. of a 24-hr. subculture on Loeffler's serum agar. The mice died 4 days after inoculation.

Rift Valley Fever.—The strain was maintained by passage of infected liver. Mice were inoculated intraperitoneally with infected liver suspension; they died 4 days after inoculation.

Intravascular Haemolysis without Infection

Groups of 10 mice were injected intravenously with preparations which caused or simulated intravascular haemolysis and gave rise to haemoglobinuria. The preparations included haemoglobin solution, lysed mouse red cells, washed rabbit red cells, saponin and phenylhydrazine.

Collection of Urine

Urine was collected in a glass metabolism cage from groups of 10 to 50 mice. Faeces were separated with a terylene net screen, or by means of a polythene funnel with a curved spout similar to the glass funnel described by Brittain (1959). Urine was usually collected in a bottle containing 0.05 ml. of N HCl, and was neutralized to *p*H 7.2 before testing. In some experiments the collecting vessel was immersed in alcohol and solid carbon dioxide so that the urine was frozen as soon as it reached the bottle. Mice were fed daily for 3 hr. outside the cage; the rest of the time between inoculation and death was spent in the cage; the urine collected was not contaminated with food. Water was unrestricted at feeding time; in the metabolism cage it was provided in a bottle or 1 ml. was given daily into the stomach of each mouse to provide a good urine flow. Collected specimens of urine were stored at $-15°$. In some experiments, urine was collected with a pipette directly from the mouse after gentle pressure on the abdomen. In some experiments the urine was passed through alumina columns (Savory and Moore, chromatographic grade) before testing.

Preparation of Extracts

Blood and urine samples were collected in siliconed apparatus. Extracts of urine, blood and tissues were prepared by Code's modification (1937) of Barsoum and Gaddum's (1935) method for the assay of histamine.

Urine was dialysed for 24 hr. in a cellophane sac against an equal volume of water and the fluids on both sides of the membrane tested. Urine was also dialysed against running water for 24 hr.

Extracts of blood and urine were also prepared with hot ethanol. Two volumes of boiling absolute ethanol were added to the sample, and boiling continued for 10 min. The liquid was centrifuged and filtered and the filtrate evaporated to dryness under reduced pressure at less than 50°. The residue was dissolved in a small amount of glacial acetic acid and precipitated by the addition of 9 vols. of anaesthetic ether. The precipitate was washed with ether, dried and dissolved in de Jalon solution.

A crude separation of peptides was made by the method of Gaddum and Horton (1959). 25 ml. of urine of mice infected with *Babesia* was passed through a column (10×1 cm.) of amberlite IRC 50 resin adjusted to *p*H 6.0. The liquid which passed through the column was reserved. The column was washed with phosphate buffer at *p*H 6.0 and then adjusted to *p*H 9.0 by the addition of the calculated amount of N NaOH. Elution was then carried out with M phosphate buffer at *p*H 9.0 and serial fractions of 2.5 ml. of eluate were collected. Part of each fraction was used for test and part was evaporated to dryness, extracted with hot ethanol, and the extract evaporated and dissolved in de Jalon solution for test. All samples and extracts were adjusted to *p*H 7.2 before test.

Isolated Organs

Guinea-pig ileum was suspended in a bath of Tyrode solution at 37°. Rat duodenum was prepared by the method of Horton (1959) and suspended in a 3 ml. bath of de Jalon solution at 31°. Atropine (10^{-6}) was used for both preparations to depress spontaneous movements. Atropine, mepyramine, triprolidine, eserine, iproniazid, bretylium and lysergic acid diethylamide were tested as antagonists, usually at a concentration of 10^{-4} in the bath.

Enzymes

Urine specimens and hot ethanol extracts were incubated for 1 to 24 hr. at 37° with proteolytic enzymes and the effect on the response of the rat duodenum tested. Controls were set up containing all reagents except the enzyme and containing normal mouse urine instead of infected mouse urine.

Chymotrypsin.—0.5 mg. was added to 0.25 ml. of sample in 0.1 M $NaHCO_3$ buffer.

Trypsin.—10 mg. was added to 0.25 ml. of sample in 0.1 M $NaHCO_3$ buffer.

Pepsin.—5 mg. was added to 0.5 ml. of sample in N/10 HCl.

Papain.—10 mg. was added to 0.5 ml. of sample in buffer at *p*H 6.0 containing 2 mg. of cysteine.

Samples of urine were also heated with N/10 NaOH in the boiling water bath for 10 min. and neutralized. Their activities were compared with unheated samples containing equivalent amounts of salt.

Haemoglobin was determined in urine by the acid haematin method.

Potassium was determined in blood and urine in the flame photometer.

Results

It seemed possible that liberation of histamine might be responsible for shock in infected animals, and accordingly an attempt was made to determine the histamine content of urine, blood, skin, nose and feet of rats and mice during the course of infection with *B. rodhaini*. It was soon apparent that the urine and tissues of infected animals contained substances which interfered with the assay of histamine. An example is shown in Fig. 1; an extract of blood which contained no detectable histamine caused a slow contraction of the guinea-pig ileum which persisted on washing out. Sensitivity to subsequent standard doses of histamine was greatly increased.

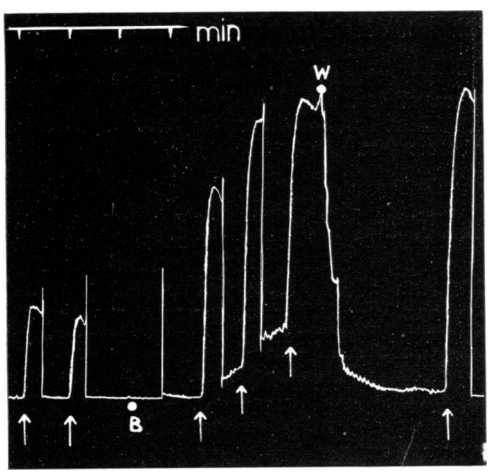

FIG. 1.—The effect of an extract of blood from mice infected with *Babesia* on the response of the guinea-pig ileum to histamine. Tyrode solution containing atropine 10^{-6}; 10 ml. bath. At arrows: 30 ng. histamine. At B: 1 ml. extract of *Babesia* blood (Code process). Wash periods: 30 sec., except at W, which was for 2.5 min.

Fig. 2 shows that hot ethanol extracts of infected blood caused larger contractions of guinea-pig ileum than an extract of normal blood.

Large amounts of active material were found in the urine of infected mice and rats. Fig. 3 shows the contractions of the guinea-pig ileum caused by a series of daily specimens of urine collected from mice during the 5 days of the infection. Fig. 4 shows that the appearance of

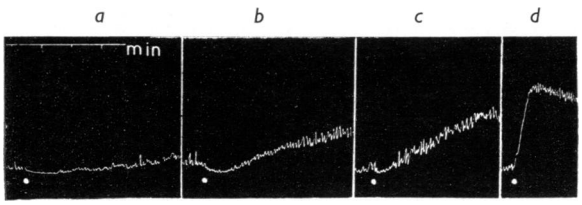

FIG. 2.—The effect of hot ethanol extracts of the blood of mice infected with *Babesia* on the isolated guinea-pig ileum. Tyrode solution containing atropine 10^{-6}; 10 ml. bath. 1 ml. ethanol extracts of mouse blood at (*a*) normal blood; (*b*) blood containing 1% of parasitized erythrocytes; (*c*) blood containing 17% of parasitized erythrocytes. At (*d*), 500 ng. histamine.

active substances in the urine preceded the rise of parasitaemia and haemoglobinuria. This occurred both in mice which died 5 days after inoculation and in rats which recovered from the infection spontaneously.

In many experiments with guinea-pig ileum, a slight relaxation or inhibition of spontaneous movements preceded the contraction caused by *Babesia* urine. The initial relaxation was much more marked when the urine was tested on the isolated rat duodenum (Horton, 1959). Relaxation was usually slight at the beginning of the infection and increased from day to day; on the day before death the relaxation was much greater than the subsequent contraction (Fig. 5).

The responses of different specimens of rat duodenum were variable. The typical reaction to *Babesia* urine was a relaxation followed by a contraction, but with some preparations relaxation predominated and, with others, contraction. The response depended partly on the load on the lever; lightly loaded preparations were more

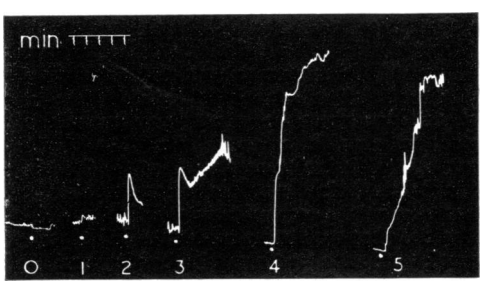

FIG. 3.—The effect of urine from mice infected with *Babesia* on the isolated guinea-pig ileum. Tyrode solution containing atropine 10^{-6}; 3 ml. bath. At 0, 0.1 ml. urine collected before infection; at 1, 2, 3, 4 and 5, 0.1 ml. urine collected on 1st, 2nd, 3rd, 4th and 5th days of the infection. The mice died after the 5th day.

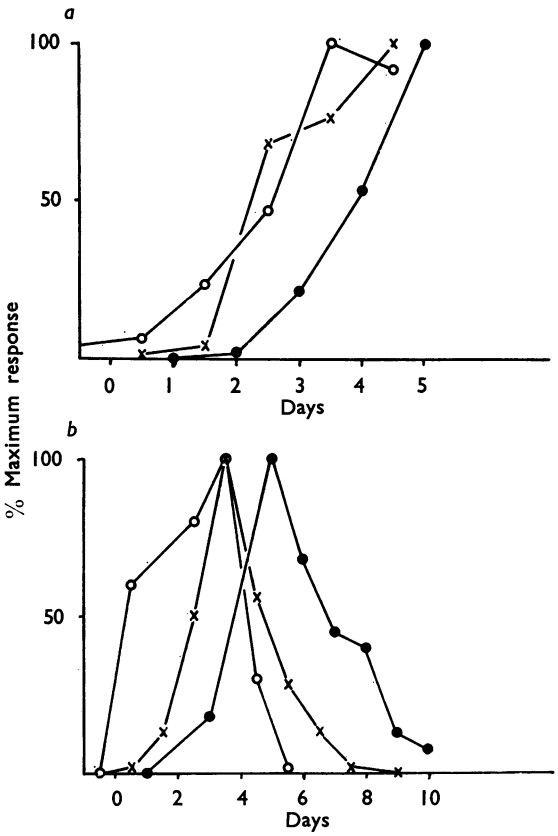

FIG. 4.—The progress of *Babesia* infections in mice (*a*) and rats (*b*) as measured by parasitaemia (●——●), haemoglobinuria (X——X) and the activity of the urine on the isolated guinea-pig ileum (O——O). Measurements are plotted as percentages of the maximum. The maximum percentage of parasitized erythrocytes in mice was 94% (5th day) and in rats 42% (6th day). The maximum degree of haemoglobinuria in mice was 21.2 mg./ml. and in rats 7.5 mg./ml. The effect on the ileum was measured as the height of the contraction produced in experiments similar to that illustrated in Fig. 3.

likely to relax well and more heavily loaded preparations were more likely to contract. A load of 0.5 g. as recommended by Horton (1959) was found to be the optimum for showing both responses.

Normal mouse or rat urine sometimes gave a small contraction and sometimes a small relaxation; the effect was never as large as that of *Babesia* urine. Urine collected from mice inoculated intraperitoneally with normal mouse blood showed no increase in active substances.

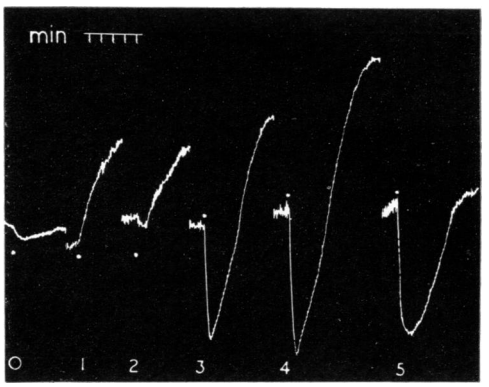

FIG. 5.—The effect of urine from mice infected with *Babesia* on the isolated rat duodenum. De Jalon solution with atropine 10^{-6}; 3 ml. bath. The samples of urine were the same as those in Fig. 3; the dose was 0.05 ml.

Bradykinin always gave a relaxation which seldom recovered as far as the original base-line until the bath was washed out.

The variable effect of *Babesia* mouse urine on the rat duodenum and the day-to-day qualitative changes which took place as the infection progressed suggested that there was probably more than one active substance in the urine. When a specimen of mouse urine which caused contraction of the rat duodenum was passed through an Amberlite IRC 50 column at *p*H 6.0 the filtrate gave a pure relaxation. When the column was eluted at *p*H 9.0 the first fraction collected caused relaxation; this fraction would be expected to contain kinins (Gaddum and Horton, 1959). The second fraction gave a contraction followed by a relaxation and subsequent fractions gave pure contractions (Fig. 6). The maximal response was given by the fifth sample collected. Hot ethanol extracts of the fractions gave responses of the rat duodenum almost identical with those of the fractions from which they were prepared. The activity of the fractions was unchanged by passage through alumina columns; the active substances passed readily through a cellophane membrane.

When urine from infected mice, or the material extracted from it by hot ethanol, was incubated with trypsin or pepsin, the activity was not destroyed in 24 hr. Papain destroyed all activity in 1 hr. Incubation with chymotrypsin for 1 hr. increased activity; incubation for 24 hr. destroyed almost all activity (Fig. 7). Boiling with alkali reduced, but did not abolish, activity. Active substances were present in urine collected

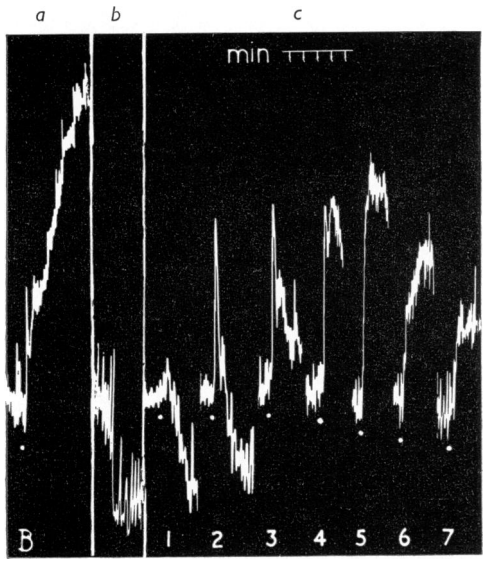

FIG. 6.—The separation of active fractions from the urine of mice infected with *Babesia*. Rat duodenum in de Jalon solution with atropine 10^{-6}; 3 ml. bath. (*a*): at B, untreated *Babesia* urine; (*b*): fluid which passed through Amberlite IRC 50 column at *p*H 6.0; (*c*): at 1–7, successive fractions collected from the column washed with buffer at *p*H 9.0. All doses 0.03 ml.

directly from the mouse by pressure on the abdomen and were stable in the refrigerator at −15°. Specimens stored in a refrigerator at 4° showed partial conversion of relaxing to contracting activity.

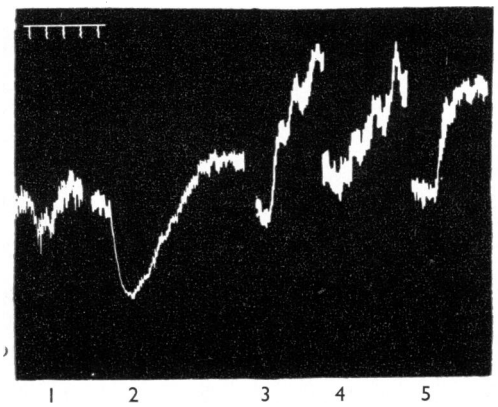

FIG. 8.—Active substances in the urine of mice in which haemoglobin has been released intravascularly. Isolated rat duodenum in de Jalon solution with atropine 10^{-6}; 3 ml. bath. Doses 0.02 ml. Time in min. 1, normal mouse urine; 2, *Babesia* mouse urine; 3, 4 and 5, urine from mice injected intravenously with lysed mouse blood, saponin, and rabbit erythrocytes respectively.

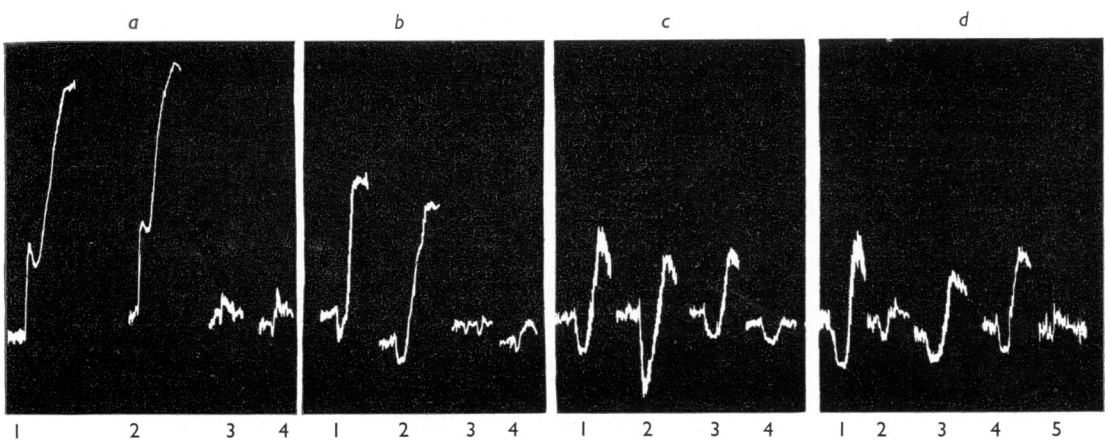

FIG. 7.—Effect of enzymes on urine of mice infected with *Babesia*, assayed on isolated rat duodenum. De Jalon solution with atropine 10^{-6}; 3 ml. bath. All doses 0.03 ml. (*a*) Pepsin: 1, *Babesia* urine incubated 24 hr. alone; 2, *Babesia* urine incubated 24 hr. with pepsin; 3, normal urine incubated 24 hr. alone; 4, normal urine incubated 24 hr. with pepsin. (*b*) Trypsin: 1, *Babesia* urine incubated 24 hr. with trypsin; 2, *Babesia* urine incubated 24 hr. alone; 3, normal urine incubated 24 hr. alone; 4, normal urine incubated 24 hr. with trypsin. (*c*) Chymotrypsin: 1, *Babesia* urine incubated 1 hr. alone; 2, *Babesia* urine incubated 1 hr. with chymotrypsin; 3, *Babesia* urine incubated 24 hr. alone; 4, *Bab sia* urine incubated 24 hr. with chymotrypsin. (*d*) Papain: 1, *Babesia* urine, fresh; 2, *Babesia* urine, incubated 1 hr. with papain; 3, *Babesia* urine, incubated 1 hr. alone; 4, *Babesia* urine, incubated 24 hr. alone; 5, *Babesia* urine, incubated 24 hr. with papain.

Experiments in which haemoglobin, lysed washed mouse erythrocytes, rabbit erythrocytes or haemolytic drugs were injected intravenously into mice showed that in all instances pharmacologically active substances were excreted in the urine. The quantities were rather less than would have been expected in *Babesia* urine showing the same degree of haemoglobinuria, and the relaxing component was apparently absent (Fig. 8). Haemoglobin, in larger amounts than those present in *Babesia* urine, when given alone or with normal mouse urine had no effect on the isolated guinea-pig ileum or rat duodenum. Urine mixed with fresh mouse blood had activity which in some instances was similar to that of *Babesia* urine.

Atropine, mepyramine, tripolidine, eserine, iproniazid, bretylium and lysergic acid diethylamide at a concentration of 10^{-4} in the bath had no effect on the relaxation or contraction of rat duodenum caused by *Babesia* urine or extracts of urine (Fig. 9). Higher concentrations of lysergic acid diethylamide had no effect on the relaxation but sometimes reduced the subsequent contraction of the rat duodenum.

Mice infected with *Plasmodium berghei, Trypanosoma rhodesiense, Streptococcus pyogenes* and Rift Valley fever virus all excreted substances in the urine which caused contraction or relaxation of the rat duodenum (Fig. 10). In acute fatal infections, the amount of relaxant material increased greatly towards the end.

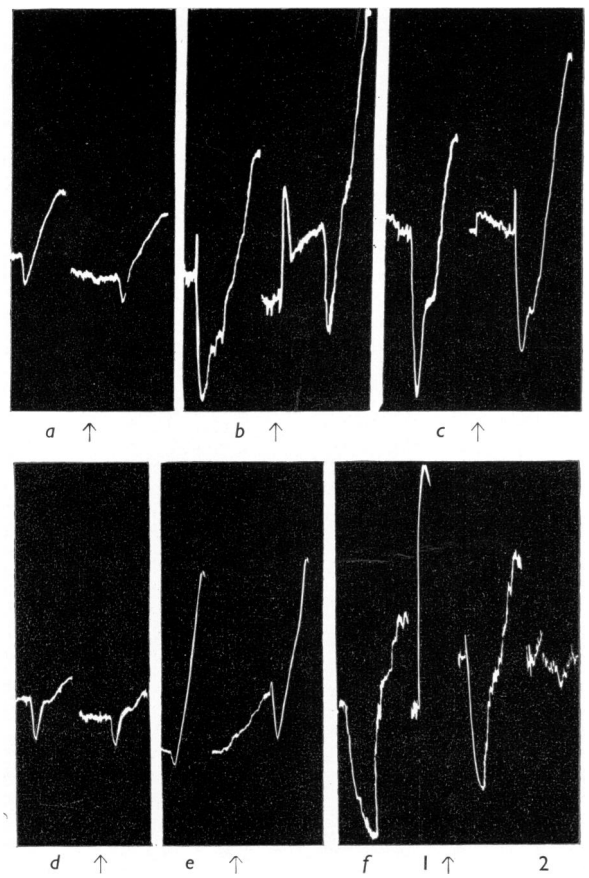

FIG. 9.—The lack of effect of various drugs on the response of the isolated rat duodenum to urine of mice infected with *Babesia*. De Jalon solution with atropine 10^{-6}; 3 ml. bath. The effect of a dose of 0.02 ml. of urine was recorded and the bath washed out. The drug was added (↑) to give a concentration of 10^{-4} in the bath and a second dose of 0.02 ml. urine then added without washing out. (*a*) Atropine, (*b*) mepyramine, (*c*) iproniazid, (*d*) eserine, (*e*) bretylium, (*f*) lysergic acid diethylamide; 5-hydroxytryptamine was given at 1 (0.005 ml. 10^{-4} solution) and at 2 (0.01 ml. 10^{-4} solution); the bath was washed out between the second dose of urine and the second dose of 5-hydroxytryptamine.

DISCUSSION

The cause of death of animals infected with protozoa is often obscure. Post-mortem examinations frequently show that the organs, although not normal, exhibit little evidence of severe impairment of function. *Babesia rodhaini* infections in mice are often heavy, and death may often be accounted for by the severity of the anaemia. However, the death of puppies infected with *B. canis,* and of mice treated with a non-toxic dose of an effective drug, cannot be explained in this way. The results of the present investigation show that the blood and urine of infected animals contain substances which are active on smooth muscle, some of which are probably peptides. They are stable to boiling with hydrochloric acid but not with alkali, they are extractable with hot ethanol, they pass readily through cellophane, they are destroyed by papain and chymotrypsin but not by trypsin or pepsin. Their action on smooth muscle is not affected by antihistamines, atropine, eserine, or by lysergic acid diethylamide in doses which abolish the action of 5-hydroxytryptamine. Identification of the active constituents will depend upon the separation of relatively pure fractions from the mixture of substances which has been shown to be present. Until this is done, quantitative parallel assays are of doubtful value.

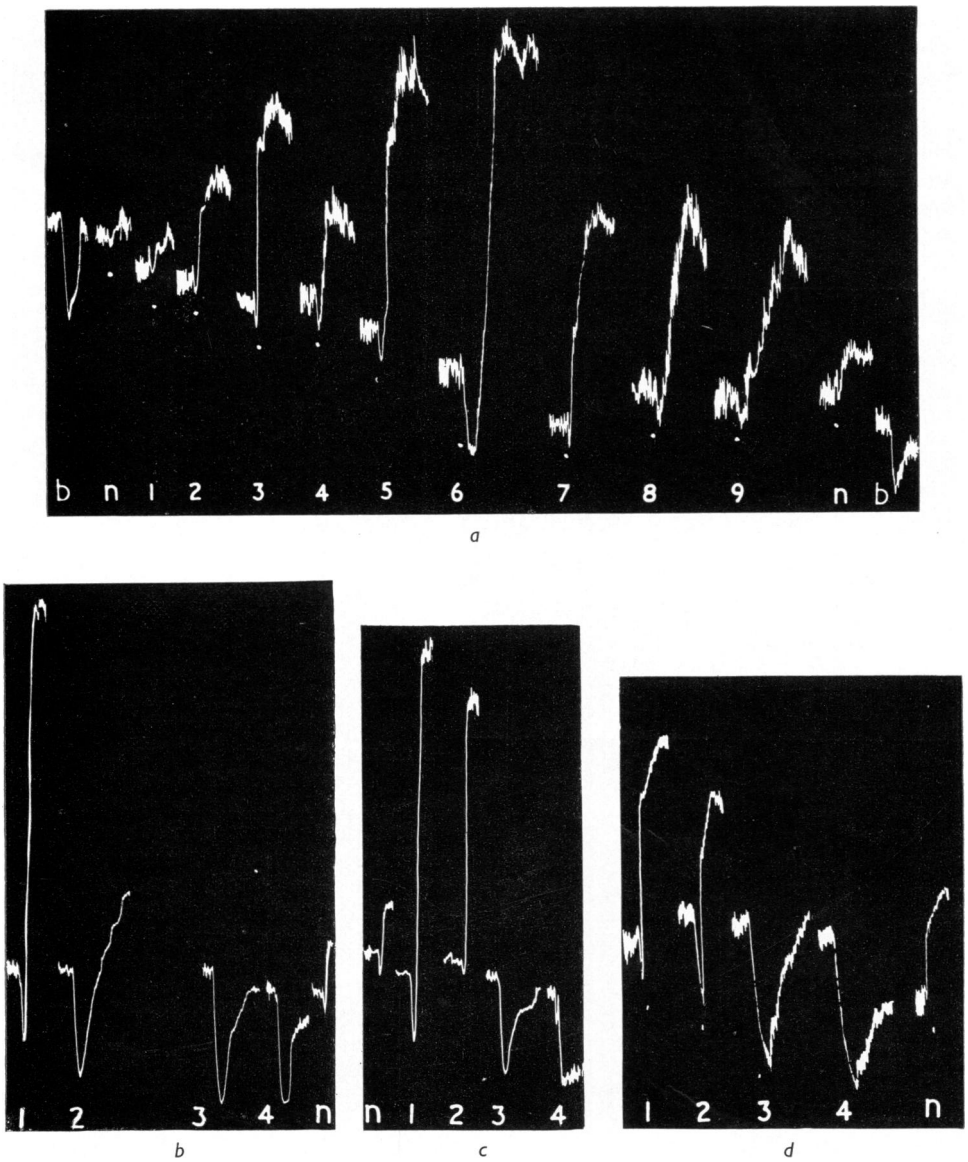

FIG. 10.—Active substances in the urine of mice infected with various organisms. Isolated rat duodenum in de Jalon solution with atropine 10^{-6}; 3 ml. bath; doses of urine 0.02 ml. b= bradykinin (10^{-6} in bath); n=normal mouse urine collected in metabolism cage; 1–9=urine samples collected daily until death. (*a*) *Plasmodium berghei*; (*b*) *Streptococcus pyogenes*; (*c*) Rift Valley fever; (*d*) *Trypanosoma rhodesiense*.

It is of interest to speculate upon the origin of the active substances which appear in the blood and urine of infected animals. *Babesia* and malaria parasites live within the red cells and destroy haemoglobin; they must possess powerful proteolytic enzymes. At schizogony, the erythrocyte bursts and the merozoites, together with their metabolic residues, are released into the plasma. It is possible that protein residues may be released from the broken erythro-

cytes and also that free enzymes derived from the parasite may attack plasma proteins and release peptides. In addition, the presence of broken cells and parasites in the plasma may start the chain of reactions which give rise to the release of kinins from plasma globulins. The experiments in which intravascular haemolysis was induced in mice by the injection of drugs or of lysed or incompatible red cells show that haemolysis is followed by the appearance of active substances in the urine.

Beraldo (1952) described substance U, a peptide in dog urine which was formed by the mixture of urine with traces of blood. He suggested that the release of substance U might explain some of the symptoms of pathological conditions in which blood and urine come into contact (Beraldo, 1955). The present experiments are clearly related to Beraldo's work and extend it into the field of the pathology of infectious diseases.

Pharmacologically active peptides occur not only in the urine of animals infected with haemolytic organisms, but also in trypanosome and virus infections. It is likely that the sensitization of guinea-pig ileum to histamine demonstrated in Fig. 1 is connected with the histamine sensitization which occurs in pertussis infections of mice (Parfentjev and Goodline, 1948). Matsui and Kuwajima (1959) have recently shown that the histamine sensitizing factor is "a toxin which has nothing to do with preventive antigens."

It seems possible that active peptides are liberated from the proteins of the host in all serious illnesses and that their pharmacological effects may be associated with the symptoms and signs of infectious disease.

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