sented. The relative intensities seem to be independent of the protein, for similar spectra were obtained with bovine serum albumin, egg albumin, and trypsin. The results in acid solution were less consistent, with the main maximum appearing at 4170 A. The phosphorescence spectra of tyrosine and tryptophane in alkaline media have been found to lie in the same wavelength region as the protein phosphorescence.

TABLE 1

PROTEIN PHOSPHORESCENCE SPECTRUM FEATURES

Wavelength (A)	Feature
4020	Marked shoulder
4180	Minor maximum
4200	Small minimum
4250	Small shoulder
4400	Broad, intense maximum
~ 4800	Cutoff

The exponential-decay phosphorescence of proteins can be attributed to tyrosine, tryptophane, and possibly phenylalanine. Since the emission is not sensitive to temperature changes, it appears to be an electronic transition. A long series of investigations was carried out by G. N. Lewis and his group on just such transitions of aromatic compounds. As the results here parallel theirs, it is assumed that the observed transitions are of the same type. The nature of this phosphorescence was reviewed by Kasha (*6*); Nauman (*5*) has studied the spectra, and McClure (*7*) investigated the transition rates. Their work indicates that the excited phosphorescent state is the lowest-lying triplet level.

A protein phosphorescence of very long duration has been observed along with the exponential-decay phosphorescence, but the mechanism is quite different. The long-lifetime emission is highly sensitive to temperature increases, and proceeds so rapidly at the dry ice temperature that it is not observed. According to Linschitz (*8*), the spectrum probably is the same as that of the exponential-decay phosphorescence. The presence or absence of this emission is a sensitive function of the pH, for it is strong in alkaline media but absent in acid media. It has been possible to study the rate of decay of the long-lifetime phosphorescence, and a report has been made elsewhere on the observed rate law and the associated mechanism (*9*).

The long-lifetime phosphorescence can be attributed to certain forms of the aromatic amino acids. Phenylalanine does not have a long-lifetime emission; tryptophane does in neutral and alkaline media, and tyrosine has this phosphorescence only in alkaline media. Further study of this phenomenon with many organic compounds has shown that the anilinium ion and free phenol forms are not active, whereas the free aniline base and phenolate ion forms are active, at least under the present conditions.

A complete description of the long-lifetime phosphorescence is not pertinent here, but a brief mention of its nature will be given, for we feel that it is related to the denaturation of proteins by ultraviolet light. The molecule absorbs a quantum of light and an electron is photo-ejected into the surrounding solid. At 77° K, the rate of return, which we believe to be diffusion-controlled, is slow; the phosphorescence occurs when the electron returns to the protein.

Harris (*10*) observed that most proteins take up oxygen when irradiated with light from the mercury-vapor arc, and that tyrosine and tryptophane absorb oxygen at a rapid rate. Gelatin (which contains little tyrosine and no tryptophane, and whose phosphorescence is much weaker than that of most other proteins) and most amino acids do not absorb oxygen.

Whether the oxygen reacts with the dissociated electron, with the protein which has lost an electron, or with the protein containing a group which has been excited into the triplet state is a question which cannot be answered with present information. It does seem quite probable that the intermediates in phosphorescence play a large part in the photo-oxidation of proteins and in their photodenaturation. Further, tyrosine and tryptophane are undoubtedly the main contributors to protein phosphorescence, both of the exponential and of the long-lifetime type.

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The Presence of Toxins other than DDT in the Blood of DDT-poisoned Roaches[1]

James Sternburg and C. W. Kearns[2]

Department of Entomology,
University of Illinois, Urbana

It has been reported by Bot (*1*) that roaches (*Periplaneta americana* L.) in the prostrate stage of poisoning after topical application of DDT may contain sufficient DDT in their blood to produce typical DDT-poisoning symptoms and death in flies (*Calliphora erythrocephala* Meig.) injected with a volume of 20 μl of such blood. Our first attempts (*2*) to duplicate the work of Bot resulted in failure, presumably because we bled roaches too early in the prostrate stage of poisoning or allowed them to approach too close to death. We have since been able to obtain samples of blood from prostrate roaches which produce the effects described by Bot, but we have not been able consistently to obtain samples with a level of toxicity that

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will produce indisputable symptoms. Part of the difficulty arises from the fact that the most toxic samples are sufficient to produce definite symptoms only when injected in volumes approaching the maximum that the housefly or the flesh fly (*Sarcophaga crassipalpis* Macq.) will tolerate if injected with blood from normal roaches.

We have chemically analyzed blood samples taken from DDT-poisoned roaches by the Schechter-Haller (*3*) method and find that they may contain from less than 1 to as much as 15 ppm DDT. These analyses were made using a microadaptation of the Schechter-Haller method, where 0.2 μg DDT can be determined accurately and amounts down to 0.05 μg detected. The method of application of DDT to the roaches influences the amount of DDT subsequently found in the blood. If 150 μg DDT is applied topically to the coxae, using 15 μl of ethanol, up to 15 ppm DDT may be found in the blood at prostration. Allowing roaches to run until prostrate in a jar containing a deposit of 50 mg DDT/1000 cm^2 yields similar results. Both these methods are such that DDT is smeared over a considerable portion of the body. If, however, dioxane is used as the solvent, and 2 μl containing 150 μg DDT is applied to the membrane between the prothoracic legs, less than 1 ppm will be present in the blood at prostration.

The toxicity of the blood does not correlate with the concentration of DDT. That is, samples of prostrate roach blood containing less than 1 ppm DDT may be equal to or more toxic than samples containing 10 or more ppm. We have also found that toxic samples of blood have about equal effects when injected into either DDT-resistant or -susceptible strains of houseflies. The particular resistant strain of flies used in these experiments could tolerate injected doses of 5 μl of normal roach blood to which DDT had been added to bring it to a concentration of 50 ppm.

Samples of toxic blood taken from DDT-poisoned roaches, extracted several times with ether or benzene, remained toxic even though controls showed that the method would quantitatively remove DDT from blood samples containing known amounts of the compound. Thus it seems that the presence of DDT in blood from DDT-poisoned roaches is not the principal factor determining the ability of houseflies to show DDT-poisoning symptoms when injected with it.

Blood drawn from roaches will ordinarily clot, because of clumping of the hemocytes. If the blood is frozen immediately upon removal from the roach, however, it will not clot when thawed. In practice, blood was collected by cutting the antennae, applying a slight amount of pressure to the roach, and allowing the blood to drip onto a watch glass resting on dry ice. From 50 to 100 roaches yield 1 ml of blood. The frozen blood was placed in a small tube, thawed, centrifuged to throw down the hemocytes, and the clear straw-colored serum was decanted off. This was refrozen and stored in this state to prevent melanin formation.

It was apparent that, in order to study the nature of the toxin in blood from poisoned roaches, a more sensitive method of detecting its presence would be required. It also seemed desirable to find a method in which the presence of small amounts of DDT, which frequently occur in the blood, would not interfere with the detection of toxins other than DDT. Roeder and Weiant (*4*) showed that DDT induces trains of impulses in the sensory nervous system but does not induce trains when applied to the central nervous system of the roach. The fact that samples of toxic blood produced typical symptoms of DDT-poisoning suggested the possibility that it might produce abnormal effects in the sensory nervous system and possibly in the central nervous system.

The effect of toxic blood on the sensory system of the metathoracic leg was investigated by the same means used by Roeder and Weiant (*4*) for DDT suspensions and emulsions. Silver electrodes were attached to the crural nerve after exposing it by cutting the membrane at the base of the metathoracic coxa. The nerve potentials were fed into a preamplifier to a cathode-ray oscilloscope, and then recorded photographically. An audio unit allowed observations to be made without continual watch of the image on the cathode-ray tube. Blood from prostrate roaches injected through the cut end of the tibia and forced

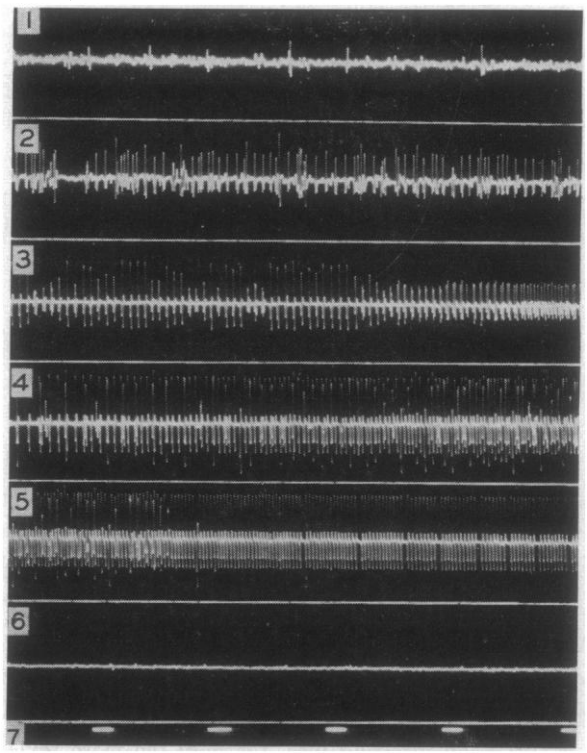

FIG. 1. Response of isolated central nervous system of American roach to blood from DDT-prostrate roaches, recorded with a cathode-ray oscilloscope: 1, normal appearance in physiological saline; 2, after 2 min in toxic blood; 3, after 5 min in toxic blood (gain reduced); 4, after 7 min in toxic blood; 5, after 7.5 min in toxic blood (gain further reduced); 6, after 8 min in toxic blood; 7, time signal, 0.1 sec between marks.

through the leg so that it appeared within the coxa caused high-frequency trains to appear within a few minutes. Blood from untreated roaches was ineffective. Since 10 ppm DDT can produce this effect within 2 or 3 min (*4*), it might be argued that the results produced by the toxic blood were due to DDT.

It was found, however, that toxic blood applied to the central nervous system would cause an abnormal response. The nerve cords used in this work were carefully dissected from adult male American roaches and placed in physiological saline. Observations of the normal activity of each cord were made by placing it in saline in a shallow groove of a plastic block, and attaching silver electrodes between the second and third abdominal ganglia. The entire unit was kept in a plastic box in which the relative humidity was near 100% to prevent drying of the preparation. After making certain that the cord was normal, the saline was drawn off, and replaced with the blood sample. Blood from untreated roaches, or from roaches treated with DDT but not yet showing poisoning symptoms, had no effect. If blood from roaches prostrate from DDT-poisoning is placed on an isolated nerve cord, an immediate increase in the normal spontaneous activity occurs, and within a few minutes high-frequency impulse trains appear. These increase in number and duration and finally become almost continuous. In most cases a sudden block of the activity follows, lasting from 1 to 15 min, after which a gradual return to normal may occur. This sequence of events is typically illustrated in Fig. 1. The more toxic samples of blood cause the build-up of trains, followed by a sudden block, to take place within 3 min. These effects are not dependent upon the amount of DDT present. Toxic samples of blood containing less than 1 ppm DDT are capable of causing this effect.

It is apparent that this method will permit the measurement of the relative amounts of toxin present even though there is considerable variation in the pattern of spontaneous activity from one nerve cord to another. The effect of toxic blood becomes unmistakably apparent by the marked increase in activity, even though it may be for a short duration in cases where the blood sample may have a low level of toxic material. The method has the added advantage of reflecting only the stimulating effects of toxins in the blood other than DDT, unless samples containing relatively large amounts of added DDT (20 ppm) are allowed to remain on the cords for periods of approximately ½ hr or longer.

It would be logical to assume that the toxic products found in the blood of DDT-poisoned roaches gradually accumulate in the blood as the symptoms of DDT-poisoning progress. It is not possible to test this assumption by injecting flies, for only the more toxic samples from prostrate roaches produce any detectable symptoms, and they may actually be the result of a combination effect with small amounts of DDT. Roaches bled in various stages of DDT-poisoning ranging from hyperexcitable to prostrate do display the progressive accumulation of a toxin in their blood,

TABLE 1

RELATIONSHIP OF STAGE OF POISONING IN DDT-POISONED ROACHES TO APPEARANCE OF TOXIN IN BLOOD

Stage of poisoning	Effect of blood on isolated nerve cord
Hyperexcitable	Cord 1: No effect.
	Cord 2: A few bursts of high activity at 6 min, then normal again.
	Cord 3: A few trains at 6 min, then back to normal.
Early prostration	Cord 4: Activity up at once. Trains at 3 min; blocked at 4 min.
	Cord 5: Activity up at once. Trains at 8 min; gradual return to normal.
	Cord 6: Activity up at once. Trains at 6 min; blocked at 10 min.
Late prostration	Cord 7: Activity up at once. Trains at 20 sec; blocked at 1 min, then came back, and repeated cycle.
	Cord 8: Activity up at once. Trains at 2 min; blocked at 7 min.
	Cord 9: Activity up at once. Trains in 1 min; blocked at 18 min.

as shown by the observations recorded in Table 1.

Vinson and Kearns (*5*) have shown that roaches injected with certain dosage levels (4–12 μg/roach) of DDT may be rendered prostrate if held at a temperature of 15° C, and revived to normal behavior if transferred to 35° C. Similar results may be obtained by topical application of 75 μg DDT to the ventral membranous area of the prothorax. This suggested testing blood from roaches prostrate at the low temperature to see if it was toxic, and subsequently testing blood from roaches of the same group after revival at the high temperature to see if it lost its toxicity. The results of this experiment are shown in Table 2, where it will be seen that roaches prostrate

TABLE 2

CORRELATION OF THE EFFECTS OF TEMPERATURE ON THE REVERSIBILITY OF DDT-POISONING SYMPTOMS AND THE ABILITY OF ROACH BLOOD TO STIMULATE THE SPONTANEOUS ACTIVITY OF THE ISOLATED ROACH NERVE CORD

Time and temperature sequence before roaches were bled					Condition of roaches when bled	Effect of blood on isolated nerve cord
35°C	15°C	35°C	15°C	35°C		
20 hr					Normal	None
20 "	1 hr				Prostrate	Trains
20 "	2 "				"	"
20 "	4 "				"	"
20 "	4 "	½ hr			Intoxicated	None
20 "	4 "	1 "			Excitable	"
20 "	4 "	2 "			Normal	"
20 "	4 "	2 "	1 hr		Prostrate	Trains
20 "	4 "	2 "	2 "		"	"
20 "	4 "	2 "	4 "		"	"
20 "	4 "	2 "	4 "	½ hr	Intoxicated	Slight increase activity
20 "	4 "	2 "	4 "	2 "	Normal	None
20 "	4 "	2 "	4 "	2 "	Prostrate	Trains

at the low temperature contain a high level of toxin in their blood, which disappears after they become normal on transfer to the high temperature. The reverse is also true. DDT-treated roaches apparently normal at 35° C contain blood that will not excite the isolated central nervous system, but roaches from the same group rendered prostrate with DDT-poisoning by transfer to 15° C contain blood that will produce a high level of stimulation in the isolated nerve cord.

At present we are studying the nature of the toxin produced in the blood of DDT-poisoned roaches, the role of temperature in relation to the disappearance of the toxin, and the presence of the toxin in relation to the mechanism of action of DDT. The results of these studies will be reported in greater detail later.

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Differentiation between Circulins A and B and Polymyxins A and E by Paper Chromatography

J. H. Dowling, Henry Koffler, H. C. Reitz, D. H. Peterson, and P. A. Tetrault[1]

Departments of Chemistry and *Biological Sciences, Purdue University, Lafayette, Indiana*, and *The Upjohn Company, Kalamazoo, Michigan*

Circulin, a mixture of antibiotics produced by *Bacillus circulans* Q-19, consists of basic polypeptides that are biologically and chemically closely related to the polymyxins (*1–7*). In fact, Peterson and Reineke (*2*) reported that the circulin fraction that they studied most intensively (since then designated as circulin A) had qualitatively the same composition as polymyxins A and E. All three antibiotics were thought to contain L-threonine, D-leucine, L-α, γ-diaminobutyric acid (DABA), and an optically active isomer of pelargonic acid with the properties of 6-methyloctanoic acid (*8*). Unlike polymyxin A, however, circulin was inactivated in the presence of lipase. These workers had no polymyxin E available for comparative work and were therefore unable to rule out the possibility that circulin A and polymyxin E were the same.

[1] Many colleagues gave us invaluable help in this work, and we gladly express our thanks to the following: George Brownlee, formerly of the Wellcome Research Laboratories, Beckenham, Kent, Eng., for sending us a sample of polymyxins A and E, and for arranging with Tudor Jones to compare circulin and polymyxin E by paper chromatography; and Harold Nash, of the Pitman-Moore Company, Indianapolis, Ind., for his continuous cooperation and permission to use some of his unpublished data in this paper.

Peterson and Reineke obtained circulin A after repeated chromatography over a mixture of equal amounts of Darco G-60 and Celite 545, using 25% aqueous tertiary butanol adjusted to pH 4.0, with sulfuric acid as the developing solution. This system separated crude circulin into two main components, namely, fraction A and the more rapidly moving circulin B. Using a combination of the above procedure and paper chromatography with the system to be described later, we obtained preliminary evidence that strain Q-19 probably produces, in addition to the two major components already mentioned, at least three other ninhydrin-positive, biologically active entities of as yet unknown nature. This paper records the fact that circulins A and B can be distinguished from polymyxins A and E by paper chromatography.

Preparation of the circulins. Separation of circulin A from circulin B was accomplished by the procedure mentioned above, or by the following method suggested by Nash (*9*): Impure circulin sulfate was dissolved in a minimal amount of *n*-butanol that had been saturated with a 0.1 *M* sodium citrate–hydrochloric acid buffer (pH 2). This solution was added to a column of Celite 545, which had been moistened by the buffer saturated with *n*-butanol. The developing agent was *n*-butanol saturated with buffer. The fractions collected were extracted twice with 10-ml portions of distilled water. After the biological potency of each extract was determined (*cf.*[*1*]), the appropriate extracts were pooled and concentrated *in vacuo* to a small volume. An acetone solution of picric acid was used to precipitate the biologically active material from an aqueous solution. The picrate was converted to the hydrochloride by gaseous hydrogen chloride or concentrated hydrochloric acid.

Polymyxins A, D, and E. Polymyxin A was approximately 48% pure, and its hydrochloride assayed 4800 polymyxin A u/mg. Polymyxin E regarded as essentially pure was furnished in the form of a base, and its sulfate assayed 11,600 polymyxin E u/mg before it was converted to the free base with gaseous ammonia at pH 8.2. Polymyxin D was used as hydrochloride and contained 1280 polymyxin D u/mg.

Paper chromatography. The antibiotics (usually 100 μg in 5 μl) were applied to Whatman No. 1 filter paper strips and permitted to dry. Unless otherwise indicated, the solvent system used consisted of the following: 49.5% *n*-butanol, 49.5% water, and 1.0% glacial acetic acid (*9*). The strips were hung for descending chromatography in an airtight glass cylinder and were allowed to equilibrate for about 2 hr with the vapors from the aqueous phase of the solvent system. The nonaqueous phase was then used to develop the chromatogram. Development continued for at least 60 hr. Ninhydrin was used to indicate the position of the peptides. Duplicate strips served to make certain that the ninhydrin-positive materials were actually antibiotically active. After chromatography the strips were placed for 8 min on the surface of an agar medium that had been seeded with *Escherichia coli ATCC 26* (*cf.* [*1*]), and then removed.