Crystallized hemoglobin in *Rhodnius prolixus* after a blood meal on guinea-pig[1,2]

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Summary. Several blood-sucking arthropods, after a blood meal, are able to store the hemoglobin from their hosts in a crystalline state in their digestive system[5,15,20]. Guinea-pig hemoglobin crystallizes in the stomach of the reduviid bug *Rhodnius prolixus* in two different crystal types. We show them to be crystallographically identical and to contain the same liganded state of hemoglobin, i.e. they represent different habits of the same crystal modification. The hemoglobin crystallizes in oxy-form and ages in the crystalline state, first to aquo-methemoglobin and subsequently to hemichrome without crystal cracking. The rate of aging appears to be the same for both types. The hemoglobin crystal modification observed in the digestive system of *Rhodnius prolixus* is highly host- but not parasite- specific. The same modification is also observed in vitro and in *Ornithodorus moubata*, an arachnid whose digestive system differs considerably from that of the insect *Rhodnius*. The retainment period of the crystals represents a long term host-record of possible medical interest.

Rhodnius prolixus Stål (Heteroptera, Reduviidae), a main vector of Chagas' disease, is found in Central and the northern part of South America[9]. Blood is the only nutrient the insect needs for molting, metabolism and egg production[8].

Insects, originally from San Salvador, were held at 25–26 °C and 80% relative humidity, and were fed monthly on guinea-pigs (*Cavia porcellus* L.). After a 3–4-h period following feeding, the blood in the stomach, i.e. the anterior storing part of the midgut, was concentrated by resorption and extraction of a quantity of fluid equal to about 40% of the blood meal weight[12]. Within 4 days after the meal most erythrocytes were hemolyzed and the hemoglobin crystallized. This phenomenon was observed in all developmental stages of *Rhodnius prolixus*[7]. The crystal size seems to depend on the developmental stage of the insect[14]; crystals with a size up to 0.2 mm are found in adults. Two crystal types can be observed (fig. 1, right side: top and middle); the typical guinea-pig hemoglobin pseudo-tetrahedral shaped crystal (we call it type T) and a flattened pseudo-tetrahedral shaped crystal (type F). Type F can be either exclusively present, as is sometimes the case in young larval stages, or can be mixed with the mostly dominant type T crystals. Their bright red color and their shape led us to characterize them tentatively as guinea-pig hemoglobin crystals, whose morphology is known[19,20].

Characterization of crystals

Adult and larval stages of *Rhodnius prolixus* were dissected in 0.7% NaCl solution. The in vivo grown crystalline material from the stomach (fig. 1, left side) was then transferred in a stabilizing standard 3 M ammonium sulphate solution buffered with 0.1 M potassium phosphate at pH 6.7 prior to crystallographic and spectroscopic analyses. X-ray precession photographs taken from single type T and F crystals showed these types to be crystallographically the same orthorhombic crystal modification with different habits. Their space group is $C222_1$ (extinctions are observed for $h+k$ odd for general *hkl*- and *l* odd for *ool* reflections), with cell dimensions $a=8.45\pm0.005$ nm, $b=8.99^5\pm0.01$ nm, and $c=8.31\pm0.005$ nm. Only the presence of 4 hemoglobin tetramers (mol.wt 64,500 daltons each) per unit cell leads to an acceptable V_M-value[13,20] (2.45×10^{-3} nm^3 per dalton). Consequently[11,20], the hemoglobin tetramers must lie on crystallographic twofold axes, their local dyads coinciding with crystallographic

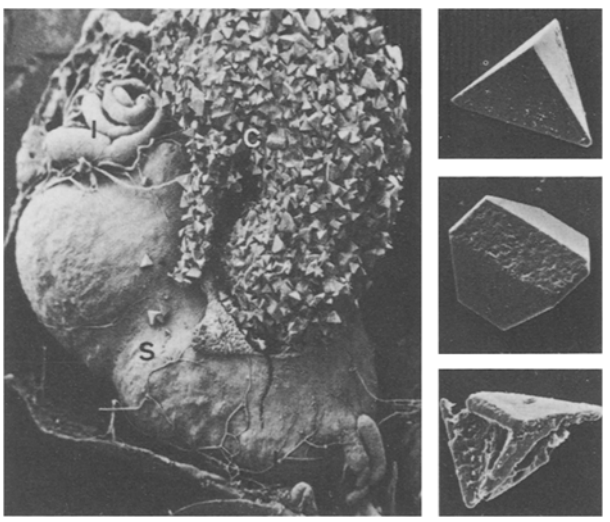

Figure 1. Appearance of in vivo grown guinea-pig hemoglobin crystals in the stomach of *Rhodnius prolixus*. Left side: Scanning electron micrograph (× 15) of the midgut of larval stage III (6 days after blood meal). Hemoglobin crystals (C) protrude from an artificial hole in the stomach (S); I, intestine. Right side: Scanning electron micrographs of characteristic hemoglobin crystals.
Top: Type T, orthorhombic disphenoid (× 315).
Middle: Type F, combination of orthorhombic disphenoid and pinacoid (× 430).
Bottom: Partially degraded type T crystal (× 220) with marked surface irregularities, taken 1 month after blood meal. Preparation of material: fixation in 6% glutaraldehyde, dehydration to 100% acetone, air dried, sputter coated with 30 nm Au.

dyads parallel to either the *a* or *b* axis of the crystal. The in vivo grown crystals from *Rhodnius prolixus* are thus identical with the in vitro grown crystals which we obtained earlier[20] from the purified major hemoglobin component (~ 80% of the total hemoglobin) of guinea-pig blood by membrane bound dialysis against concentrated ammonium sulphate solutions. Visual comparison of precession photographs from in vivo and in vitro grown crystals did not show any significant differences in intensities between them. This clearly proves that the crystals found in *Rhodnius prolixus* contain guinea-pig hemoglobin. The observed types T and F simply represent different habits of the same crystal modification; type T crystals are pure orthorhombic disphenoids with {111} or {$\bar{1}\bar{1}\bar{1}$} faces whereas type F crystals are a combination of an orthorhombic disphenoid and a pinacoid with either {100}, {010} or {001} faces.

The different habits of the crystallographically identical type T and F crystals could have been induced by a preferential incorporation of different liganded forms of guinea-pig hemoglobin during crystal formation. Therefore we recorded transmission spectra from 700 to 400 nm of the visually different type T and F crystals. The spectra were recorded immediately after dissection of the animals to avoid changes in the momentary Fe^{2+}/Fe^{3+} ratio of the crystalline hemoglobin. Figure 2 gives the calculated absorption spectra of type T and F crystals taken from stage II and stage IV larvae, 7 and 12 days after a blood meal respectively. All crystals contain a mixture of oxy-hemoglobin, with absorption maxima at 540 and 577 nm, and aquo-methemoglobin with maxima at 500 and 631 nm. The spectra of equally old type T and F crystals show nearby identical compositions irrespective of the developmental stage of *Rhodnius*. The spectra also show that the hemoglobin crystals age in vivo, i.e. the relative amount of aquo-methemoglobin increases with time elapsed after the blood meal. This aging process and its progress are not dependent on the crystal type, implying that the formation of T and F type crystals is not governed by specific incorporation of different liganded states of hemoglobin.

Crystal fate

The in vivo aging process of guinea-pig hemoglobin crystals was followed in greater detail by dissecting larvae at different time intervals, from 3 days to 75 days, after the blood meal and subsequent recording of spectra from crystals. From these spectra (fig. 3) we conclude that guinea-pig hemoglobin crystallizes in *Rhodnius* in its oxy-form within days after a blood meal (spectrum 3 days). The oxy-hemoglobin turns within weeks into aquo-methemoglobin (12- and 30-day spectra) and then gradually (spectrum 75 days) into hemichrome with its characteristic absorption maximum[18] at 535 nm and accompanying shoulder at 565 nm. The spectrum of the 75-day-old crystal still represents a mixture of aquo-methemoglobin and hemichrome. Its hemichrome content is obvious from the shallower absorption minima around 480 and 600 nm, the reduced absorption at 630 nm, and the appearance of new maxima at 535 and 565 nm in comparison to the nearly pure aquo-methemoglobin spectrum of the 30-day-old crystal (fig. 3). The changes in iron oxidation state and ligandation during transformation from oxy-hemoglobin via aquo-methemoglobin into a hemichrome structure in the solid phase are compatible with the observed crystal lattice, since the crystals do not show physical damage such as cracking on aging. Moreover, they can still be recognized as being of the T or F type. The aging of guinea-pig hemoglobin crystals in vivo seems to be a more general phenomenon as it is also observed in the tick *Ornithodorus moubata*[20]. Aged crystals still diffract well[20] and they show no significant changes in cell parameters.

The crystals remaining in the stomach 1–2 months after a blood meal, often show strong irregularities (fig. 1, bottom right). This could either signify a resorption process in a changed gut environment or

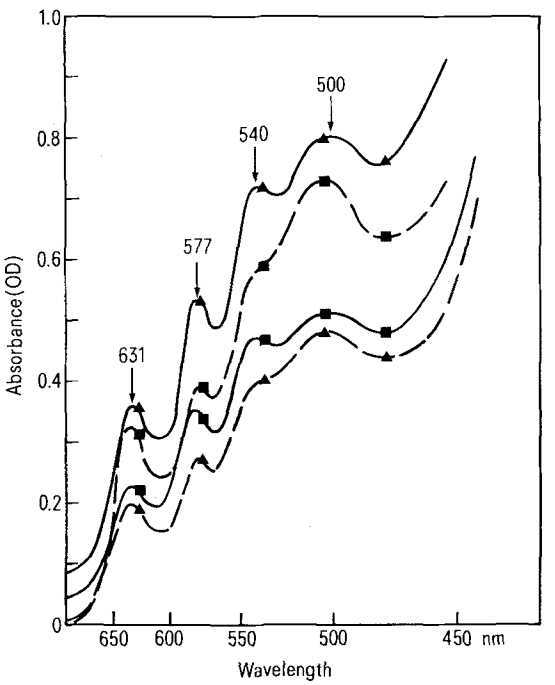

Figure 2. Absorption spectra from single in vivo grown type T and type F hemoglobin crystals. Type T (—▲—) and type F (—■—) crystal from the stomach of larval stage II of *Rhodnius prolixus*, 7 days after a blood meal on guinea-pig. Type T (--▲--) and type F (--■--) crystal from the stomach of larval stage IV, 12 days after meal. Spectra were recorded on a Zeiss universal microspectrophotometer UMSP I. The wavelength scale is given by the dispersion of the quartz monochromator (M4QIII). Solvent was 3 M ammonium sulphate, 0.1 M potassium phosphate, buffered at pH 6.7. Specific absorption maxima[22] occur for oxy-hemoglobin at 540 and 577 nm, for aquo-methemoglobin at 631 and 500 nm.

be caused by proteases which, by means of a backflow mechanism, could move from the intestine to the stomach[6]. In the intestine, i.e. the digestive posterior part of the midgut, crystals can be detected only near the stomach region. There, they are probably subject to quick digestion by intestinal proteases[6].

The main cause for the crystallization of hemoglobin in the digestive system of blood-sucking arthropods seems to be the concentration of the blood meal by fluid resorption and excretion[5,17,20]. This creates a supersaturated hemoglobin solution from hemolyzed erythrocytes which crystallizes by itself in the gut environment. According to Korzhuev (cited by Balashow[5]), hemoglobin crystals derived from a single blood sample may differ in form if crystallization is induced by different methods. The habitat of crystals and other residues from earlier blood meals may also affect the crystal-formation from new blood meals. Moreover, host intoxication may lead to changed crystal forms[16]. Possibly some of these causes, except host intoxication, play a role in the relative abundance of type T and F crystals as observed in *Rhodnius prolixus*. As shown above, differences in the liganded state of the hemoglobin certainly do not determine the T/F ratio.

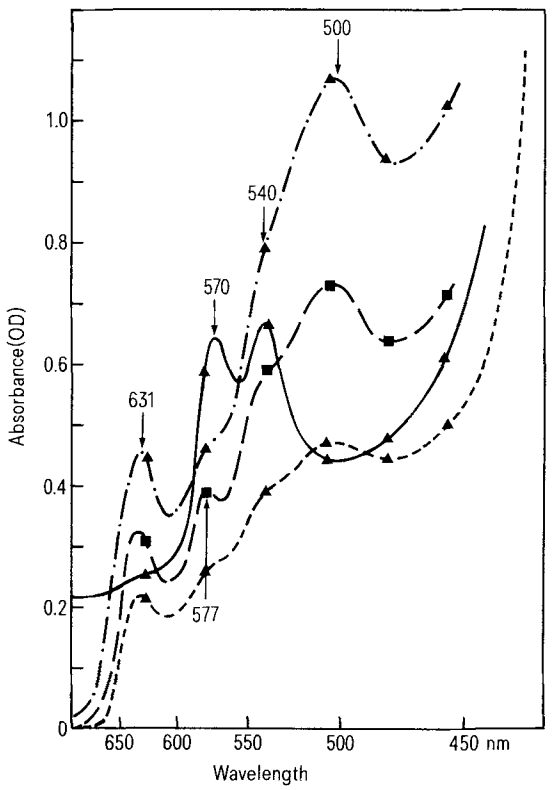

Figure 3. Absorption spectra from single in vivo grown hemoglobin crystals at different time intervals after a blood meal on guinea-pig.
Type T crystal from stomach of larval stage III, 3-day (—▲—); standard solution saturated with CO before recording of the spectrum.
Type F crystal, larval stage IV, 12-day (--■--).
Type T crystal, larval stage IV, 30-day (-·▲·-).
Type T crystal, larval stage III, 75-day (--▲--).
Conditions as in fig. 2. Specific absorption maxima occur for oxy-hemoglobin[22] at 540 and 577 nm, for carbomonoxy-hemoglobin[22] at 540 and 570 nm and for hemichrome[18] at 535 nm with a shoulder at 565 nm.

Host specificity

The phenomenon of in vivo crystallization of hemoglobin has also been described for other reduviid bugs, bed bugs, ioxid and argasid ticks and occasionally for tsetse flies and mosquitos[5,15,20]. Obviously, the crystalline state is an efficient method of nutrient storage, which is widely used by bugs and ticks.

We have shown here that two habits of the same crystal modification exist in all developmental stages of the reduviid bug *Rhodnius prolixus*. Moreover, this modification is crystallographically identical both with those of hemoglobin crystals observed in the tick *Ornithodorus moubata* after a blood meal on guinea-pig and in vitro grown crystals[20]. *Rhodnius* and *Ornithodorus* belong to different classes of the phylum Arthropoda and they differ widely in their digestive system too. The digestive tract of *Rhodnius*, a hexapod, includes stomach, intestine, rectum and anus[6]. In contrast, *Ornithodorus*, an arachnid, possesses merely a dead-end stomach as the connection to the neighboring intestine is non-functional in physiological terms[10]. Despite these differences both animals produce the same crystal modification from guinea-pig hemoglobin. Hence, the hemoglobin crystals found in the digestive system of blood-sucking arthropods appear to be highly host- and not parasite-specific, despite occasional differences in their habit. The long retainment period for these host-specific hemoglobin crystals in the gut of reduviid bugs and ticks allows the identification of earlier hosts[5,15,20,21]. Their identification is easily performed by optical inspection and axial analysis of the in vivo found hemoglobin crystals. This is equally true for aged crystals, because of the observed constancy in their unit cell parameters with time. Thus, this long-term host record of blood-sucking arthropods, which are mostly vectors of tropical diseases, might prove to be of considerable medical interest.

1 Dedicated to Prof. Rudolf Geigy on his 80th birthday.

2 Acknowledgments. We thank Dr R. Richle for providing *Rhodnius prolixus*, Dr R. Halonbrenner for recording transmission spectra, H.P. Giuliani, A. Hefti and M. Düggelin for taking electron micrographs and Proff. S. Graeser and K.H. Winterhalter for discussions and support.

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0014-4754/83/121335-04$1.50 + 0.20/0

Short Communications

Hyperthermia after intrahypothalamic injections of thyrotropin releasing hormone (TRH) in the pigeon[1]

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Summary. Thermoregulatory responses to intrahypothalamic injections of thyrotropin releasing hormone (TRH) were recorded from unanesthetized pigeons exposed to 6 °C, 20 °C and 32 °C. Our results suggest that TRH is a non-specific excitatory neuromodulator or neurotransmitter for heat production in the pigeon.

There is a considerable amount of evidence showing that TRH is distributed not only in the hypothalamus but also throughout the nervous system[3–5]. Besides stimulating the release of thyrotropin (TSH) from the adenohypophysis, TRH is also known to affect prolactin secretion[4,6].

Several studies have confirmed the thermoregulatory effects of TRH in mammals[3,4]. However, similar effects are not well known in birds. Intracerebral administration of TRH has been shown to elevate the body temperature (T_b) in the fowl by activating heat production and decreasing thermodispersive mechanisms[7].

The aim of the present study was to determine the effect of intrahypothalamic administration of a large range of different dosages of TRH on temperature regulation in the pigeon. In addition, the effects of season and various ambient temperatures (T_a) on the thermoregulatory responses were considered.

Materials and methods. Using pentobarbital anesthesia, a guide cannula was implanted stereotaxically[8,9] into the brain of the pigeon *(Columba livia)*, with the tip located either in the preoptic (PO/AH) area or in the posterior hypothalamus. 22 birds weighing 275–425 g were used in the study. 10 pigeons were used in November (group A), 5 with the guide cannula in the PO/AH area and 5 with the

Effects of intrahypothalamic injections of TRH on shivering in pigeons at ambient temperatures used in the winter (W) and in the spring (S)

T_a (°C)	Dosage (ng)	Season	Shivering (μV) Before injection	Maximum increase	
6	100	W	29.0±5.67	9.0±1.20	×
	200	W	31.5±8.89	12.0±1.99	×
	200	S	22.5±7.96	12.0±3.24	×
	500	W	27.1±7.14	13.7±3.46	×
	500	S	20.3±5.96	9.5±2.60	×
20	50	W	14.6±5.44	12.3±1.35	×
	50	S	11.8±3.07	7.5±1.85	
	100	W	21.3±5.53	18.8±6.20	×
	200	W	12.8±2.90	8.8±2.24	×
	200	S	7.8±3.71	14.5±3.62	×
	500	W	19.0±6.47	24.4±5.55	×
	500	S	7.8±3.12	11.7±2.21	×
32	100	W	0	11.8±5.08	×
	200	W	0	6.3±2.88	×
	200	S	0	4.8±0.95	×
	500	W	0	8.1±3.33	×
	500	S	0	4.3±1.03	×

Values are mean±SE. × Significant difference ($p<0.05$ or less) compared with corresponding controls (Student's t-test).