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Protease Activity in Female *Ornithodoros tholozani* Ticks

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Abstract

The protease activity in guts of *Ornithodoros tholozani* females was studied *in vitro*. The intracellular protease in *Ornithodoros tholozani* guts has a pH optimum of about 3.0. Hemoglobin is the preferred substrate, and bovine serum albumin is digested very slowly. In this respect the protease resembles cathepsin D.

Unfed ticks contain a small amount of protease in the gut. After feeding the level of protease increases gradually for several days until peak protease activity is attained. The level of gut protease activity depends on the size of the blood meal taken and on the interval after feeding. After a period of peak protease activity, the level of protease declines. The level of gut protease in unmated females (kept at 27°C) did not reach prefeeding levels within 100 days.

The level of gut proteolytic activity, as determined by *in vitro* protease assays, does not reflect the degree of blood digestion which takes place *in vivo*. After a period of rapid digestion, lasting for about two weeks, the undigested part of the blood meal remains unchanged in the lumen of the gut. At that time the gut tissue contains considerable levels of protease, which can be demonstrated by *in vitro* assays. Presumably, the protease remains active inside the gut cells, although the uptake of hemoglobin from the gut lumen has ceased.

The results are compared to those obtained in other tick species.

Introduction

The digestion of hemoglobin in ticks is intracellular (BALASHOV, 1972), and thus differs from blood digestion in insects, which takes place in the lumen of the intestine (GOODING, 1972).

Histological studies demonstrate that in ticks the lysis of erythrocytes takes place in the lumen of the gut (TATCHELL, 1964), but the hemoglobin is degraded inside the digestive cells (BALASHOV, 1972; GRANDJEAN & AESCHLIMANN, 1973). Biochemical studies of the digestive enzymes of ticks also indicate that digestion is intracellular, since the proteolytic enzymes in the guts of the ticks have an optimum pH of about 3.0, whereas the pH of the gut contents is 6.5 (TATCHELL, 1964; TATCHELL et al., 1972; BOGIN & HADANI, 1973).

In ticks the processes of feeding, mating, digestion and egg development are interrelated and are essentially different in the two families *Argasidae* and *Ixodidae*. The differences, as summarized by AESCHLIMANN & GRANDJEAN (1973), are related to the fact that the ixodid females engorge only once, develop eggs and die after oviposition, whereas in argasid females feeding, digestion, vitellogenesis and oviposition are cyclic activities which may be repeated several times.

In *Ixodidae* the female does not engorge prior to mating. After mating the female takes one huge blood meal and digestion starts. The digestive cells of the

midgut epithelium are synchronized, i.e. all the cells are in the same state of activity at the same time. The nutrients of the blood meal are used up to produce one large egg mass, and the female dies after laying the eggs.

In *Argasidae* mating in nature usually occurs after feeding. Virgin females feed normally, but egg development is very slow, or does not occur at all. After feeding the rate of digestion increases for several days. If mating does not occur, digestion is slowed down. The rate of digestion is more rapid in mated than in unmated female *Ornithodoros tholozani* (GALUN & WARBURG, 1968) and *Argas persicus* (TATCHELL, 1964).

In virgin ticks, the undigested portion of the blood meal remains in the midgut diverticula and serves as a nutrient reserve which allows the tick to survive for a prolonged period, and which is mobilized for vitellogenesis when mating occurs (AESCHLIMANN & GRANDJEAN, 1973).

In *O. moubata* (GRANDJEAN & AESCHLIMANN, 1973) numerous active cells appear in the gut epithelium about 2 days after the blood meal; this phase continues up to the start of oviposition by the mated females. After oviposition the number of active gut cells declines; this phase may continue for many months. In *O. moubata* the cycles of the different digestive cells are not synchronized, so that nearly all the stages may be found simultaneously. The absence of synchronization allows a slow digestion of the hemoglobin reserves, enabling the tick to survive a prolonged period of starvation.

The purpose of the present study was to determine the levels of protease activity in unmated *O. tholozani* females. Several parameters were examined – such as the *in vitro* properties of the protease, the level of gut protease at various intervals after feeding, and the relationship between protease activity, the size of the meal and the amount of undigested blood in the gut lumen. Our results were compared with those obtained in other tick species.

Materials and Methods

Experimental animals

Unmated female *Ornithodoros tholozani* were obtained from individually kept blood-fed nymphs, and fed on a rat twelve months after their last nymphal blood meal. The average weight of the starved ticks was 14.6 mg ± 4.62 (s.d.). Each tick was weighed immediately after dropping off the rat, in order to minimize the loss of weight due to loss of coxal fluid (GALUN & WARBURG, 1968). The engorged ticks were kept individually at 27°C until dissection. At the appropriate interval after feeding (1–100 days after the blood meal), the gut was dissected out and kept at –20°C in 0.3 ml of 0.85 % NaCl. The rest of the carcass, including the ovary, was kept similarly in a separate vial.

Protease activity determinations

Immediately before the protease assay each gut was homogenized in cold 0.1 M glycine–HCl buffer, pH 2.95, in a glass teflon tissue grinder. The final volume of the homogenate was 2.5 ml per gut. Protease activity was determined by a modification of the assay for cathepsin D (MEDNIS & REMOLD, 1972). The amount of homogenate taken for each determination varied according to the stage of the ticks 0.3 ml of homogenate (corresponding to 0.12 gut) from unfed ticks; 0.05 ml from engorged ticks with high protease activity (at about 10 days after feeding). Routine protease determinations were done without added substrate (the

contents of the gut serving as substrate) and with crystalline bovine hemoglobin as substrate. The final volume of the reaction mixture was 1.2 ml, and it consisted of the appropriate amount of gut homogenate, 0.1 M glycine – HCl buffer pH 2.95, with or without hemoglobin (6 mg per tube). The final pH of the reaction mixtures with added hemoglobin was 3.0–3.1.

In experiments with bovine serum albumin (BSA) as substrate, the appropriate amount of the albumin was dissolved in 0.1 M glycine – HCl buffer, pH 2.95.

In experiments on the effect of pH on protease activity of guts and of the rest of the carcass, the tissues were homogenized in cold distilled water. The substrate was hemoglobin (6 mg per tube) dissolved in the appropriate buffer. We used 0.1 M glycine – HCl buffer for the pH range 2.0–3.7 and acetic acid-sodium acetate for pH 3.7–5.5. The final pH of the reaction mixtures was checked after dissolving the hemoglobin.

The reaction mixtures were incubated for 1 hour at 39° in a water bath. The reaction was stopped by 1.8 ml of cold 10 % trichloroacetic acid. The tubes were left standing for 20 minutes in an ice bath, centrifuged to remove the precipitated protein and filtered. The controls consisted of all the ingredients except for the enzyme source and were incubated for the same period; the enzyme homogenate was added after the trichloroacetic acid. All determinations were done in duplicate.

Fluorometric assay for tyrosine

Aliquots of 2.0 ml of the trichloroacetic acid supernatant were taken for the determination of tyrosine by the nitrosonaphthol method of WAALKES & UDENFRIEND (1957). Standard curves were prepared with solutions of free tyrosine (0.2–3.2 microgram per ml) in 6 % trichloroacetic acid.

The fluorescence was measured on a Turner Model 111 fluorometer with a blue lamp No. 110–853 and primary and secondary filters 470 and 570 mμ, respectively.

Protease activity

Protease activity was given as micrograms of tyrosine released in one hour per one gut, using 0.5 % hemoglobin as substrate. Since the minimal amount of tyrosine that could be detected was 0.6 μg per tube and the fluorescence was proportional to the amount of tyrosine up to 12 μg per tube, any amount of enzyme releasing 0.6–12 μg of tyrosine in one hour could be measured.

Protein determination

The amount of protein in gut homogenates was determined by the microbiuret method of GOA (1953). The trichloroacetic acid precipitates of the gut homogenates were dissolved in 4.0 ml of 3 % NaOH, 0.20 ml of Benedict's reagent was added, the tubes mixed and the extinction read (at 330 mμ) after 15 minutes. The standard curve was prepared with hemoglobin solutions in 3 % NaOH containing 0.1–1.5 mg per 4 ml. The amount of protein is given as mg hemoglobin per gut.

Chemicals

The hemoglobin was 2 × crystallized bovine hemoglobin from Sigma, St. Louis, Mo., U.S.A., Bovine serum albumin, BSA, fraction V powder, fatty acid poor, was obtained from Pentex, Miles Laboratories.

Table 1. The effect of hemoglobin and bovine serum albumin (BSA) on tick protease activity *in vitro*

Substrate added (mg per ml reaction mixture)	Protease activity * (μg tyrosine)	% of maximal activity
No additional substrate	31	20
Hemoglobin 0.625	94	61
Hemoglobin 1.25	112	73
Hemoglobin 2.5	138	89
Hemoglobin 5.0	154	100
Hemoglobin 7.5	154	100
Hemoglobin 10	142	92
BSA 4	38	25
BSA 10	38	25
BSA 20	47	31
BSA 40	115	75

* Protease activity given as μg tyrosine released per hour per tick.

Results and Discussion

Effect of substrate, amount of enzyme and time of incubation on protease activity in vitro

The first step was to determine whether hemoglobin or serum albumin were the preferred substrate for the tick protease. The homogenates (from whole engorged females, eight days after feeding, corresponding to 0.05 tick per tube) provided less than 1 mg protein substrate per incubation (1.2 ml). The pH of the reaction was 3.0. Graded amounts of bovine hemoglobin or bovine serum albumin were added in order to determine the optimal amount of substrate. *Table 1* shows that optimal activity was obtained with 0.5–1.0 % hemoglobin. Without any additional protein substrate protease activity was low – the addition of 0.4%–2.0% BSA did not increase activity appreciably. *O. tholozani* protease is thus similar to cathepsin D rather than cathepsin E since hemoglobin is a better substrate than BSA (GREENBAUM, 1971; BARRETT, 1969).

TATCHELL (1964) found that the addition of cysteine (10^{-3} M) increased the activity of *Argas* gut proteinase *in vitro* by 60–80%. We could not evaluate the effect of thiol reagents on *O. tholozani* protease, since cysteine (0.02 M) strongly interfered with the nitrosonaphthol reaction for tyrosine.

Table 2. The effect of enzyme concentration and incubation time on the *in vitro* protease activity

Substrate added	Time of incubation (min)	Protease activity (μg tyrosine released per incubation) Amount of homogenate (equivalent to no. of guts) 0.032 gut	0.08 gut	0.16 gut
0.5 % hemoglobin	20	0.95	2.6	4.8
0.5 % hemoglobin	40	2.05	5.6	11.2
0.5 % hemoglobin	60	3.15	9.0	–
0.5 % hemoglobin	90	4.70	13.2	–
0.5 % hemoglobin	120	6.10	–	–
No substrate	60	0.60	1.6	3.9
No substrate	120	0.70	2.0	4.5

The effect of the amount of enzyme and the time of incubation was determined with graded amounts of gut homogenates (from females engorged 10 days previously) incubated for 20–120 minutes. *Table 2* shows that the amount of tyrosine released was proportional to the amount of enzyme and to the time of incubation up to two hours, in incubation mixtures containing an adequate amount of substrate (0.5% hemoglobin). Without added hemoglobin, the amount of tyrosine released was proportional to the amount of enzyme only for one hour. (In assays for cathepsin D it is particularly important to allow only a small portion of the total substrate to be digested; see BARRETT, 1969.)

Effect of pH on proteolytic activity of the gut and of other tissues

The pH optimum for the *in vitro* activity of gut homogenates was determined with hemoglobin as substrate *(Fig. 1)*. The enzyme was a homogenate pooled from guts of females which had engorged 10 days previously. The amount of enzyme per tube corresponded to 0.05 gut. The pH activity curve shows a broad optimum between pH 2.7 and 3.3. The activity declined steadily with rising pH, and reached 15% of the maximal activity at pH 5.0–5.6.

The range of optimal activity in *O. tholozani* was thus similar to that in *Argas* (TATCHELL, 1964), although we did not observe a secondary peak at pH 3.8. The maximal activity of the gut protease of *Hyalomma* (an ixodid tick) was also between pH 2.8–3.2 (BOGIN & HADANI, 1973).

The protease activity with a pH of 3.0 was not confined to the gut; the protease activity of the carcass (all the tissues, including ovaries,

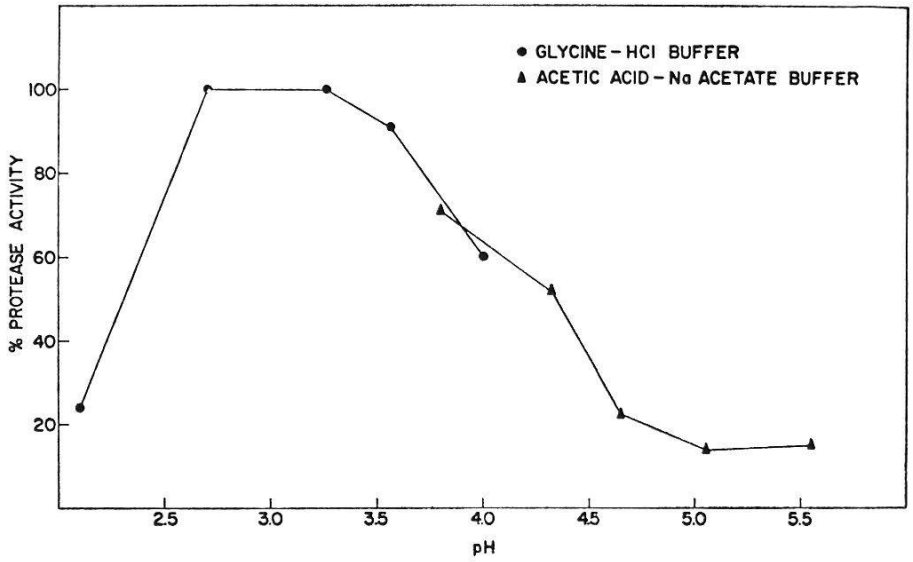

Fig. 1. Effect of pH on the proteolytic activity of homogenates from dissected tick guts.

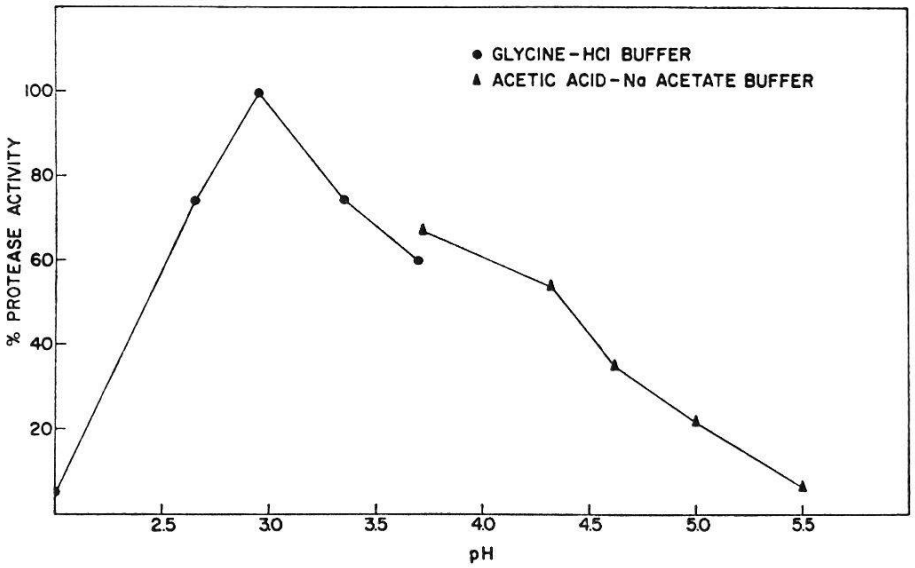

Fig. 2. Effect of pH on the proteolytic activity of homogenates from ticks after the removal of the guts.

which were left after dissecting out the gut) was considerable *(Table 3)*. The protease in the carcass had a pH optimum similar to that of the gut protease *(Fig. 2)*.

Because of the protease activity in the carcass we found it necessary to dissect the tick and use only the gut. Our results thus differed from those obtained by TATCHELL et al. (1972), who found that at the pH optimum for gut protease there was only negligible protease in tissues other than gut, and therefore dispensed with the gut dissections and used homogenates from whole ticks.

Table 3. Protease activity in the gut and the carcass of *O. tholozani* female ticks 10 days after feeding on a rat

Weight of tick after feeding (mg)	Mean protease activity * (μg tyrosine released in one hour per gut)		
	In gut	In carcass	Carcass protease
34	57	57 average	(% of total)
38	56		50.0
51–69 (average of 6 ticks)	128.5	80	38.5
138	256	51	16.5
158	322	59	15.5

* Gut and carcass protease assayed at pH 3.0 with 0.5% hemoglobin as substrate.

Gut protease activity as related to the size of the blood meal

In many blood-feeding insects the protease activity levels are related to the meal size (GOODING, 1972; AKOV, 1972). In *Argas* ticks, the protease activity (6 days after feeding, when activity is maximal) was also related to the size of the blood meal (TATCHELL et al., 1972).

The level of gut protease activity in *Ornithodoros* (kept at 27 °C for 10 days after feeding) was likewise related to the size of the blood meal. Ticks that had taken a huge blood meal (weighing 138–158 mg after engorging) had five times more gut protease than the ticks that took the smallest meal *(Table 3)*. The protease activity of the carcass was not related to the amount of gut protease. In the ticks that had taken a small blood meal the carcass protease was about equal to that of the gut; in ticks that took huge (over 100 mg) blood meals, the carcass protease was only 16% of the total protease.

Protease activity in Ornithodoros females at various intervals after feeding

Since the amount of protease varied greatly according to the size of the meal, it was necessary to use ticks that had taken approximately the same amount of blood. We therefore took only ticks weighing 50–80 mg immediately after engorging (this corresponds to a blood meal of 35–65 mg), and measured their gut protease activity five and ten days after feeding (*Table 4*). Even within this range the gut protease activity varied according to the weight of the engorged tick. We therefore subdivided the ticks into two groups: the smaller ticks, which weighed 50–59 mg after engorging, and the larger ones, weighing 60–80 mg.

Table 4. Gut protease in *O. tholozani* females 5 and 10 days after feeding

Five days after feeding				Ten days after feeding			
Wt. of engorged tick (mg)		Protease (μg tyr)*		Wt. of engorged tick (mg)		Protease (μg tyr)*	
36		100		34		57	
40		80		**38**		**56**	
43		122		52		141	
48	n = 15	**70**		52	n = 15	168	n = 11
50	rank	105		56	rank	175	rank
51	correlation	84		57	correlation	150	correlation
53	0.690	115	n = 11	59	0.480	187.5	–0.322
58		100	rank	61		194	
59		115	correlation	62		152.5	
63		105	0.709	63		167.5	
66		100		64		82.5	
67		174		64		138	
71		141		**72**		**155**	
76		174		138		155	
78		**148**		158		322	

* Protease is given as μg tyrosine per hour per gut.

The Spearman rank correlation coefficient (M_S) was computed for the data in *Table 4* (SIEGEL, 1956). This coefficient for all the ticks 5 days after feeding was 0.690 (i.e. significant at the $p = 0.01$ level for $n = 15$), indicating that the amount of gut protease at this interval after feeding is related to the amount of blood taken. When only the 11 ticks with weights 50–80 mg were taken ($n = 11$), the Spearman rank correlation was 0.709, significant at the $p = 0.05$ level. On the other hand, no positive correlation existed at 10 days after feeding in the group of ticks weighing 52–72 mg after feeding ($n = 11$, correlation – 0.322). This is also shown clearly in *Fig. 3*. Ten days after feeding the level of protease was similar in the heavier (60–80 mg) and in the lighter ticks (50–59 mg). If the extreme values were included, ticks which took very small meals (34–38 mg) or very large ones (138 to 158 mg), the Spearman rank correlation was 0.480 ($n = 15$); i.e. significant at the $p = 0.05$ level. Thus, the relationship between meal size and gut protease activity holds true only at certain time intervals after feeding *(Fig. 3)*.

Fig. 3 shows the level of gut protease at various intervals after feeding in the heavier ticks (60–80 mg) and in the smaller ones (50–59 mg). One day after feeding the level of protease was almost twice of that in unfed ticks (see also *Table 6*). On day one there was no difference in protease activity level between the two groups, but on day 5 after

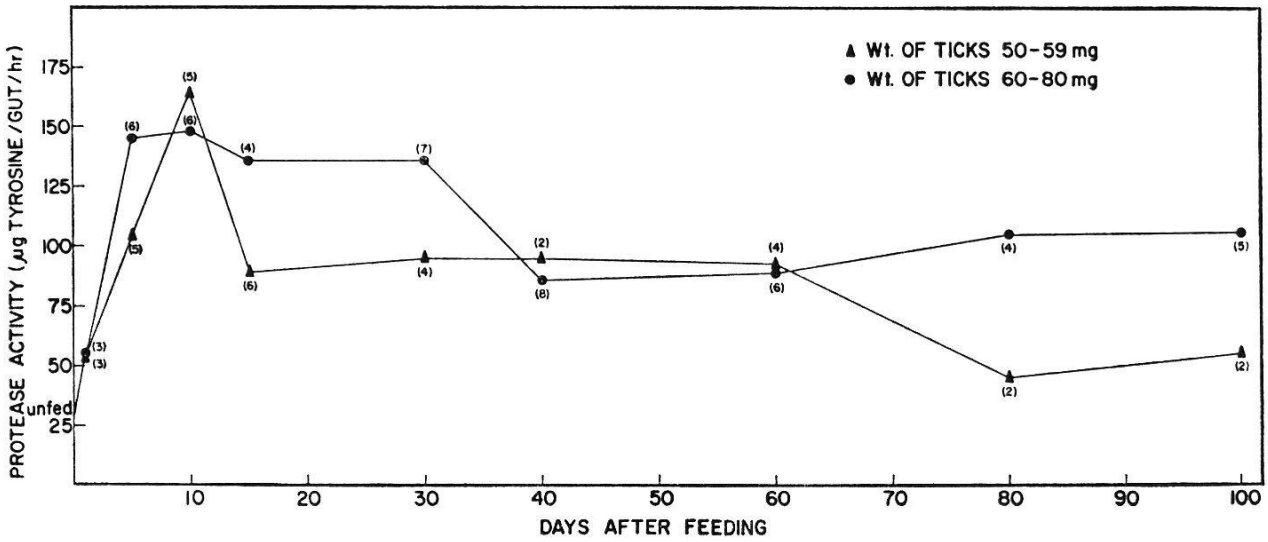

Fig. 3. In vitro protease activity of tick guts at various intervals after blood feeding. (The number of ticks dissected is in parentheses.)

feeding, the heavier ticks had more gut protease than the smaller ones. The level of maximum protease activity in the guts of the heavier ticks was not higher than that in the smaller ticks, but it was attained earlier (day 5) and maintained for a longer period (30 days) than in the latter. At 40 days after feeding it dropped to a lower level and remained unchanged until 100 days after feeding, when the experiment was terminated. In the smaller ticks the period of maximal activity was shorter, activity dropped to a lower level at 15 days, and then again at 80 days after feeding. However, protease levels 100 days after feeding were still above the level in unfed ticks.

Relationship between weight of unfed ticks and the size of the blood meal

In male and female *Argas* ticks the weight of the unfed tick and the size of the blood meal were closely correlated factors influencing the amount of protease (TATCHELL et al., 1972).

In order to determine whether the weight of the starved *O. tholozani* influences the amount of blood ingested, we weighed 39 unfed females individually and grouped them according to their weight. Of these ticks, 36 took blood meals on a rat. *Table 5* shows no correlation between the weight of the unfed tick and the weight of the blood meal taken. The ticks used in the experiment in *Table 5* had been starved for only 6 months since their last nymphal meal, whereas the ticks used in the protease experiments had been starved for a whole year. The difference in prefeeding weight between the two groups was slight; ticks starved for 6 months had an average weight of 16.9 mg (n = 39, range

Table 5. The effect of prefeeding weight on the size of the blood meal *

Prefeeding weight (mg)	Number of ticks fed	Average post-feeding weight (mg)	Average meal taken (mg)
10–12	4	72.3	61.3
13–14	7	97.9	84.4
15–16	8	102.6	87.1
17–18	6	93.5	76.0
19–20	5	112.2	92.7
22–23	**6**	**95.2**	**72.2**
Total	36	96.9 ± 16.4 (st. deviation)	80.2

* The virgin females were starved for 6 months from the time of their last nymphal meal. They were grouped according to their weight, allowed to feed on a rat, and weighed immediately after dropping off the animal.

10–23) as compared with an average weight of 14.6 mg (±4.62 s.d., n = 15, range 8–23 mg) in ticks starved for 12 months.

The length of starvation prior to the first adult feeding had little effect on the weight of the unfed tick, but it markedly decreased the size of he blood meal taken. After 12 months of starvation the average weight of the engorged female was only 71.11 mg (n = 376) as compared with 96.9 mg (± 16.4 s. d.; n = 36) after 6 months of starvation.

Physiological changes after blood feeding

GALUN & WARBURG (1968) found that in *Ornithodoros tholozani* the blood meal taken was followed by a continuous increase in oxygen uptake which reached a peak within 8–12 days and coincided in time with the intensive proliferation and growth of the digestive cells. Similar histological changes occurred in the digestive cells of another species, *Ornithodoros moubata* for several days after feeding (GRANDJEAN & AESCHLIMANN, 1973). It seems, therefore, that the increase in metabolism is related at least partly to the massive proliferation and growth which takes place in the gut epithelium, and to the intensive protein synthesis activity, which also includes the synthesis of proteolytic enzymes.

Increase in protease activity after feeding

Table 6 shows the level of gut protease in unfed ticks and the protease levels one day after feeding. The protease activity is given for each individual tick. Protease activity was measured with 0.5% hemo-

Table 6. The protease level in guts of unfed ticks and in ticks one day after feeding

	Wt. before feeding (mg)	Wt. after engorging (mg)	Protease activity (μg tyr) *			
			With 0.5 % hemoglobin		No substrate added	
Unfed ticks	9		35		2.0	
	10		32	average	0.8	average
	12		33	36.0 μg tyr	1.75	3.22 μg tyr
	15		38		6.25	
	19		43		5.4	
Ticks one day after feeding		54	49		19.5	
		54	64		26.6	
		59	46	53.5 μg tyr	20.9	23.5 μg tyr
		61	58		23.75	
		65	71		37.5	
		66	35		12.5	

* Protease activity is given as μg of tyrosine released in one hour by one gut.

globin as substrate, and also without any added substrate. In unfed ticks the average proteolytic activity per gut was 36.0 μg tyrosine per hour in the presence of 0.5% hemoglobin. In the absence of added hemoglobin substrate, protease activity in the gut homogenates from the unfed ticks was negligible. (There was no trace of blood in the guts of the unfed females which had starved for a whole year since their nymphal meal.)

One day after feeding the average protease activity per gut was 53.5 μg tyrosine per hour (with 0.5% hemoglobin substrate); an increase of 75% in protease as compared with prefeeding levels. In gut homogenates to which no substrate was added the protease activity was 23.5 μg tyrosine per hour per gut, a seven-fold increase in protease.

Effect of gut content on protease activity in vitro

The protein of the gut, which is derived from the blood meal and included in the whole gut homogenates, can serve as substrate for *in vitro* protease activity assays. In order to obtain reliable results with guts which differ greatly in their blood contents it is mandatory to provide optimal concentrations of protein substrate in all reaction vessels. With suboptimal protein the amount of protease in unfed ticks, which contain no blood in their guts, will appear to be lower than it is actually. Protease assays using homogenates from guts dissected after feeding would include the blood from the meal, and thus would contain more adequate amounts of protein substrate. The increase in protease

Table 7. The effect of bovine serum albumin (BSA) and hemoglobin on the *in vitro* protease activity of gut homogenates from unfed ticks and ticks one day after feeding on a rat

Gut homogenate *	Protease activity (μg tyrosine released in 1 hour per tube)			
	Substrate added			
	None	0.75 % BSA	3.0 % BSA	0.5 % hemoglobin
0.3 ml from unfed tick	0.2	1.4	1.5	3.6
0.3 ml from tick 1 day after feeding	2.2	2.2	2.2	5.4
0.3 ml from unfed tick + 0.3 ml from tick 1 day after feeding	3.2			

* One gut homogenized in 2.5 ml.

activity *in vitro* would be falsely attributed to a sudden increase in proteolytic enzyme due to feeding.

In order to test the role of the gut contents as substrate for protease assays, we prepared homogenates from pooled guts of unfed ticks, and from guts of ticks dissected one day after feeding on a rat. The guts were assayed for protease activity *in vitro* (using an equivalent of 0.12 gut per incubation) with the following substrates: a) no substrate added; b–c) 0.75% and 3.0% BSA, respectively; d) 0.5% hemoglobin. The gut homogenates from unfed and fed ticks were also combined, 0.12 gut from each, without added protein *(Table 7)*. The guts of the unfed ticks contained no endogenous homoglobin. The amount of hemoglobin in the fed ticks was approximately 10 mg hemoglobin per gut (see *Table 8*). The amount of endogenous hemoglobin contributed by 0.12 gut was 1.2 mg hemoglobin per reaction mixture of 1.2 ml. This amount of endogenous hemoglobin was suboptimal: the addition of 0.5% hemoglobin increased the protease activity from 2.2 μg tyrosine/tube to 5.4 μg tryosine/tube. The addition of 0.75% and 3.0% BSA to guts from fed ticks did not increase the *in vitro* protease activity because the tick protease digests BSA much more slowly than hemoglobin.

Gut homogenates from unfed ticks which contained no hemoglobin had negligible protease activity when no substrate was added *(Table 7)*. The addition of 0.75% or 3.0% BSA increased activity but less effectively than 0.5% hemoglobin. Combining the homogenates from unfed and fed ticks resulted in more protease activity than the expected

additive effect: instead of the expected 2.4 μg tyrosine (0.2 μg + 2.2 μg), the activity was 3.2 μg tyrosine, indicating that the gut content of the fed tick served as substrate for the enzyme of the unfed tick.

TATCHELL et al. (1972) obtained an immediate increase in protease activity after feeding, and they concluded that in unfed *Argas* ticks the protease is present in the form of an inert, readily activated precursor molecule. This interpretation implies that ticks are unique in the animal kingdom in possessing a cathepsin-like enzyme in the form of a pro-enzyme, since no activators of endocellular catheptic endopeptidases have been recorded in the literature (GREENBAUM, 1971). A simpler explanation is that the sudden increase in the *in vitro* protease activity was due to an increase of hemoglobin substrate since hemoglobin is the preferred substrate for tick protease. TATCHELL et al. (1972) used 0.75% BSA as the main substrate for unfed ticks, whereas the substrate for fed ticks was provided by their gut contents. We calculated from their data that an average blood meal (12 mg) on a chicken would provide at least 1.5 mg of hemoglobin (ALBRITTON, 1952), because the ticks were weighed after coxal fluid emission. Since 2 males per ml were used, the concentration of hemoglobin derived from the gut contents was 0.3%, which probably was a much better substrate than 0.75% BSA.

The fact that TATCHELL et al. (1972) used BSA, rather than hemoglobin, as the substrate for the *in vitro* digestion might also explain why these authors did not detect any protease activity in *Argas* tissues other than gut. Using BSA as substrate would not affect the results in unfed ticks with empty guts. In engorged ticks the substrate for the gut protease would be the hemoglobin from the gut contents, whereas the substrate for the protease in tissues other than gut would be the serum albumin. Since BSA is digested at a much slower rate than hemoglobin, the protease activity of the carcass tissues appeared much lower than the activity in the gut.

Preliminary experiments in our laboratory with *Argas persicus* ticks have shown that the *in vitro* protease activity can be increased by the addition of hemoglobin. When 0.2% hemoglobin was added to whole body homogenates from fed *Argas* ticks (the homogenate contained 0.125% hemoglobin from the gut contents), the protease activity was doubled. These results are very similar to those obtained with *O. tholozani* homogenates (see *Table 1*).

Proteolytic activity in vitro *and proteolysis* in vivo

The proteolytic activity reported in this study has been determined by *in vitro* assays, using whole gut homogenates. The act of homogenizing brought the contents of the disrupted gut cells in contact with a

Table 8. Amount of protein (given as mg of hemoglobin) in the guts of female *O. tholozani* ticks at various intervals after feeding. Weight of ticks immediately after feeding – from 50–80 mg

Day after feeding	Number of ticks	mg hemoglobin per gut
1	6	10.0
5	6	9.65
10	5	8.40
15	10	6.50
20	5	6.50
30	11	6.85
40	8	6.70
60	10	5.85
80	6	5.90
100	7	5.95

great excess of hemoglobin, in the presence of a buffering system, which maintained the pH required for maximal proteolytic activity. Clearly, the level of proteolytic activity, as determined by *in vitro* assays, does not necessarily reflect the true levels of protein digestion *in vivo*. SMITH & BIRT (1972), who studied the proteolytic activity during the metamorphosis of the blowfly, also concluded that *in vitro* protease assays were of doubtful value in assessing the extent of proteolysis *in vivo*.

Measuring the amount of protein in the gut contents is a more reliable method to estimate the extent of digestion in ticks. In unmated *O. tholozani* females over 50% of the blood proteins were digested within one month after feeding; during the second month an additional 10% of the hemoglobin disappeared (GALUN & WARBURG, 1968). In the present study we measured the protein contents of the guts (tissue and contents) at shorter intervals. *Table 8* shows that about one third of the protein was digested within two weeks after feeding, but very little digestion took place during the next three months. The discrepancy between our present results and those of GALUN & WARBURG (1968) may be explained by the difference in methodology. In the former study the hemoglobin in the gut contents was measured, whereas in the present study we included the gut tissue and determined the total protein. It is possible that some of the digestive proteolysis was obscured by the reutilization of the released amino acids and their reincorporation in the proteins of the gut cells, especially during the first stage of rapid digestion and gut cell proliferation (about 15 days in *O. tholozani*). (In nymphal *Argas* ticks, labelled tyrosine, added to the blood meal, was rapidly incorporated into the cytoplasm of the digestive cells, see KHALIL, 1971.) During the second phase of digestion (15–100 days) the amount of protein in the gut remained stable. The

hemoglobin in the gut lumen was not in contact with the intracellular proteases since the amount of proteolytic enzymes present in the gut cells would certainly suffice to digest the gut contents completely.

Digestion in *Argas persicus* is more rapid than in *O. tholozani.* The first rapid phase of digestion in unmated *Argas* females occurred during the first week after feeding, and there was no further decline in hemoglobin for the following three weeks (TATCHELL, 1964). However, the level of gut protease, as measured *in vitro,* remained high for a whole month after feeding in the unmated *Argas* females (TATCHELL et al., 1972). The unmated females, which did not digest the blood, had more protease activity than mated females, which digested the blood meal more rapidly.

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