Oxygen uptake during tanning of *Globodera rostochiensis*

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SUMMARY

Oxygen uptake during tanning of females from two populations of *Globodera rostochiensis* (Newcastle, NCL and Ayrshire, AYR) was monitored by standard Warburg respirometry. Substantial oxygen uptake, termed endogenous oxidation, occurred in the presence of phenylthiourea (PTU), an inhibitor of the tanning enzyme. It was unaffected by changes in pH from 6.0-7.2, showed an exponential response as temperature increased from 5-37°, and increased markedly in the presence of catechol. When females from the two populations were compared the oxygen uptake was frequently different. Causes of this endogenous oxidation are unknown. Oxygen uptakes due to the activity of the tanning enzyme were little affected by changes in either pH (6.0-7.2) or temperature (5-37°) and did not differ between NCL and AYR females. The uptakes increased markedly in the presence of catechol and differed when females from the two populations were compared over the range 1-10 mM catechol. The tanning process of cyst-nematodes can be quantified by measuring oxygen uptake.

RÉSUMÉ

Absorption d'oxygène pendant le brunissement des kystes de Globodera rostochiensis

L'absorption d'oxygène pendant le brunissement des femelles de deux populations de *Globodera rostochiensis* (Newcastle, NCL et Ayrshire, AYR) a été étudiée à l'aide du respiromètre de Warburg. Une absorption importante, appelée oxydation endogène, a eu lieu en présence de phenylthiourée (PTU), un inhibiteur de l'enzyme du brunissement. Elle n'a subi aucun changement quand le pH passait de 6,0 à 7,2, a répondu de façon exponentielle quand la température augmentait de 5 à 37° et a augmenté sensiblement en présence de catechol. La comparaison entre les femelles des deux populations a révélé de fréquentes différences dans l'absorption d'oxygène. Les causes de cette oxydation endogène sont inconnues. L'absorption d'oxygène due à l'activité de l'enzyme de brunissement n'a été que peu affectée par les mêmes changements de pH (6,0-7,2) ou de température (5-37°) et n'a pas différé d'une population à l'autre. L'absorption augmentait sensiblement en présence de catechol et, quand la concentration en catechol passait graduellement de 1 à 10 mM, des différences étaient observées entre les réactions des femelles des deux populations. Il est possible de quantifier le processus de brunissement en mesurant l'absorption d'oxygène.

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The ability of female cyst-nematodes (Heteroderidae) to tan their cuticle to form a cyst protecting the enclosed eggs is unique in the Nematoda. Colour changes that occur during tanning of individuals of the golden potato cyst-nematode, *Globodera rostochiensis*, and factors that affect these changes, have been documented (Ellenby & Smith, 1967 ; Awan & Hominick, 1982). However, assessing tanning by observing colour changes is subjective and subtle differences in degree and rate of tanning cannot be detected. Since the tanning process depends on a phenoloxidase EC 1.14.18.1 (Ellenby, 1946) which catalyzes the oxidation of phenols to quinones using aerobic oxygen, it can be quantified by measuring oxygen uptake during tanning (e.g. Hackman & Goldberg, 1967). This communication describes oxygen uptake, measured by standard Warburg respirometry, during tanning of *G. rostochiensis* females from two populations. The objectives of the experiments were to determine whether Warburg respirometry was a suitable method for objectively quantifying the tanning process, and whether differences existed between the two populations.

Materials and methods

Cysts of *G. rostochiensis* were obtained from an infested allotment in Newcastle (NCL population) and from the farm of Mr. C. W. F. Judge, Maidens, Ayrshire (AYR population). The NCL population has been cultured in this laboratory since 1941 and the AYR population since 1972. Their pathotype was not determined. Experiments were conducted from 1973-75.

Nematodes were grown on the cultivars Majestic and Golden Wonder in a controlled environment chamber with a 16 h light (23°) and 8 h dark (15°) regime. Usually a two-week-old plant was transplanted into a pot of infested soil on a day designated Day 1. Alternatively, sprouted tubers were placed in infested soil and allowed to grow for two weeks before designating Day 1. Gold females were picked from the roots by hand on Days 58-72, when they were full of eggs at various stages of development. Comparatively few eggs contained second stage juveniles.

Enzyme activity was measured by standard Warburg respirometry. Flasks with total volumes of 5-6 ml contained 1 ml of the reaction mixture. As O_2 uptakes were the same with and without KOH in the center well, most analyses were performed in the absence of KOH. Catechol was used as the phenolic substrate for the enzyme.

Gold females, removed from the roots with an artist's brush, were placed on filter paper moistened with tap water, cleaned of adhering debris, and divided into four replicates of five to seven females of the same colour. The length (from the base of the neck to the terminus) and diameter of each were measured with a micrometer eyepiece. Nematodes were then kept on moistened pieces of filter paper until used in the assay, a period of about 15 min.

The substrate of catechol, ascorbic acid and EDTA (ethylene-diamine tetraacetic acid), dissolved in buffer and adjusted to the required pH with 10 N NaOH, was prepared immediately before each assay. Seven flasks were used in each assay as follows : *i*) Thermobarometer : 1 ml buffer ; *ii*) Two autoxidation control flasks : 0.8 ml buffer, 0.1 ml substrate and 0.1 ml distilled water ; these measured autoxidation of the medium ; *iii*) Two endogenous oxidation flasks : 0.8 ml buffer, 0.1 ml substrate, 0.1 ml 3.3 mM phenylthiourea (PTU, an inhibitor of the tanning enzyme whose action is reversible (Awan & Hominick, 1982)) and 5-7 gold females. Endogenous oxidation (O_2 uptake in the absence of phenoloxidase activity) was determined by subtracting the mean uptake of the autoxidation controls from the uptakes measured by these flasks ; *iv*) Two enzyme activity flasks : 0.8 ml buffer, 0.1 ml substrate, 0.1 ml distilled water and five to seven gold females. Phenoloxidase activity was calculated by subtracting the mean autoxidation value and the mean endogenous oxidation value from the O_2 uptakes recorded for these last two flasks.

The procedure was as follows : buffer and substrate were placed in the reaction chamber (substrate was not placed in the side arm because it frequently contaminated the centre well when the flask was tipped). Nematodes were added and crushed with a flattened glass rod. Finally, distilled water and PTU were added to the appropriate flasks. They were then attached to the manometers, put into the water bath and allowed to equilibrate, usually for 15 min. Readings were taken at 5 min intervals for one hour, and 15 min intervals for the next hour. Straight lines were fitted to the data by regression analysis. Occasionally a straight line could not be fitted and the results from that particular flask were not used.

Because the size of the females varied and the enzyme is localized in the cuticle (Awan & Hominick, 1982), O_2 uptakes were expressed as $\mu l/mm^2$ cuticle. The surface area of females was estimated from the formula SA = π dl, where d = width of the female and l = length. (SA of a sphere = $4\pi r^2$ = πd^2. Since d = l for approx. 70 % of the females, d^2 was replaced by dl in the formula).

Results

PRELIMINARY EXPERIMENTS (Fig. 1)

Yellow females, punctured in buffer or water, become light brown within thirty minutes but do not tan completely (Awan & Hominick, 1982). During this time, there is a small but measurable O_2 uptake (Fig. 1 A) which ceases after about two hours. Addition of 1 mM catechol, which restores the ability to tan completely (Awan & Hominick, 1982), caused a greatly increased O_2 uptake, but the rate decreased until the reading at 60 min (Fig. 1 B). During this time, dramatic colour changes occurred in the solutions containing catechol and females. They were deep yellow at the 10 min reading and progressed to a brown colour at 60 min. Thereafter, a black tinge slowly developed. After 60 min, the cuticles appeared brown and O_2 uptake proceeded at a slow but constant rate for up to 240 min, the longest that observations were made. When PTU was added with the catechol, tanning was prevented, the solution remained colourless and while there may have been an initial slight uptake of O_2, it quickly ceased (Fig. 1 C).

This method is unsatisfactory for measuring the activity of the phenoloxidase because the rate of O_2 uptake decreased over the first hour. The most rapid uptake was probably missed as it occurred during equilibration. The constant rate after 60 min. probably resulted from secondary reactions after the initial activity of the enzyme. For this reason, it is customary to add ascorbic acid to reaction mixtures containing phenoloxidases (Hackman & Goldberg, 1967). A large excess of ascorbic acid relative to the concentration of substrate keeps the latter reduced while the ascorbic acid is oxidized. Hence, a constant concentration of the substrate is maintained long enough to allow a precise measure of the initial velocity of the reaction. EDTA must also be added to prevent rapid autoxidation of ascorbic acid. When ascorbic acid and EDTA were added to the flasks with 1 mM catechol, the solutions and cuticles remained colourless and a uniform rate of O_2 uptake was maintained for at least 150 min. (Fig. 1 D) A straight line could then be fitted and the initial velocity of the reaction calculated. The velocity remained constant as the concentration of ascorbic acid was varied from 10-50 mM and that of EDTA was maintained at 10^{-3} that of ascorbic acid. Thus, within the range of concentrations used, the results were independent of the concentrations of ascorbic acid and EDTA.

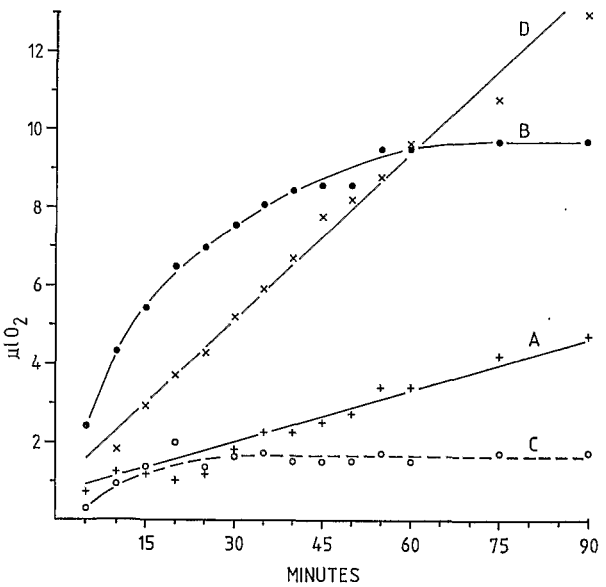

Fig. 1. Cumulative oxygen uptake by ten *G. rostochiensis* females at 25°, pH 6.8, after 8 minutes' equilibration. Females were crushed and incubated in : A, 0.09 M sodium phosphate buffer ; B, same, but 1 mM catechol also present ; C, same as B, but 0.33 mM PTU present ; D, same as B, but 50 mM ascorbic acid and 0.1 mM EDTA also present. Uptakes by control flasks containing the substances but no nematodes were subtracted before the data were plotted.

SOLUBILITY OF THE ENZYME

The tanning enzyme appears to be located in the cuticle and some, but not all, is liberated when females are punctured (Awan & Hominick, 1982). To determine if the Warburg method can measure the amounts of enzyme remaining in the cuticle, the following experiment was done at 25° with six flasks containing 0.09 M sodium phosphate buffer, pH 6.8 and 1 mM catechol, 25 mM ascorbic acid and 0.025 mM EDTA. Two flasks served as autoxidation controls. Ten females were added to each of the remaining four flasks and crushed. After agitating the flasks in the water bath for 10 min, cuticles were removed from two of the flasks and the O_2 uptake in all flasks was then monitored for two hours. If substantial amounts of enzyme remained in the cuticles, then flasks without them should show a smaller O_2 uptake compared to flasks with cuticles. Such was not the case. After allowing for autoxidation, the two flasks with cuticles had uptakes of 9.7 and 13.0 ($\bar{x} = 11.4$) compared to 10.4 and 11.1

($\bar{x}$ = 10.8) µl/mm² cuticle/min × 10^{-3} for the flasks without cuticles. Thus, the enzyme that remains within the cuticle is a comparatively small amount which is outside the limits of detection by the methods employed. Nevertheless, to save time, cuticles were left in the flasks in all subsequent experiments.

Effect of buffer and cultivar of host (Tab. 1)

The final colour of the cuticle and colour changes in the solutions were different when ACES (1(N-(2-acetamido)-2-aminoethane sulphonic acid, a zwitterion buffer) instead of sodium phosphate buffer was used (Awan & Hominick, 1982). To determine if O_2 uptakes also differed in these buffers, females from the two populations were tested in both under the following conditions : 25°, pH 6.8, 0.08 M buffer, 1 mM catechol, 25 mM ascorbic acid, 0.025 mM EDTA and 0.33 mM PTU present and absent.

The endogenous oxidation of both populations and the enzyme activity of the NCL population were significantly higher in ACES compared to phosphate buffer (t-test ; $p < 0.05$). There was no significant difference between the enzyme activities of the AYR population in the two buffers or between the autoxidation controls. Because endogenous oxidation and enzyme activity in ACES were at least as good as, and usually higher than, those in phosphate buffer, most subsequent analyses were in ACES buffer. It is also worth noting that there were significant differences ($p < 0.05$) between the endogenous oxidation and enzyme activity of the NCL and AYR females in phosphate buffer. Endogenous oxidation of the AYR females was half while their enzyme activity was almost twice that of the NCL females. These differences did not occur with ACES as the buffer.

To test if the cultivar of the host affected the results, NCL females grown on Majestic or Golden Wonder potatoes were compared. There was no significant difference between either endogenous oxidation or enzyme activity of females grown on these two cultivars (Tab. 1).

Oxygen uptake in the absence of exogenous substrate

Females punctured or crushed in the absence of catechol never tan completely, probably because the endogenous substrate for the enzyme is released and diluted (Awan & Hominick, 1982). However, there is a measurable uptake of O_2 after crushing (Fig. 1 A). To measure this uptake more accurately, AYR and NCL females were assayed under the following conditions : 25°, pH 6.8, 0.09 M ACES, 25 mM ascorbic acid, 0.025 mM EDTA and 0.33 mM PTU present and absent. The results (Tab. 2) show that about half the O_2 uptake in the absence of additional substrate can be attributed to phenoloxidase acti-

Table 1

Mean and standard deviation for the oxygen uptake by NCL and AYR females grown on cv. Golden Wonder or Majestic and incubated in 0.08 M buffer, pH 6.8, 1 mM catechol, 25 mM ascorbic acid and 0.025 mM EDTA at 25°. (N) = number of replicates.

Host	*Golden Wonder*				*Majestic*
Buffer *Nematode Population*	*Phosphate* *NCL*	*ACES* *NCL*	*Phosphate* *AYR*	*ACES* *AYR*	*ACES* *NCL*
O_2 uptake,(µl/min) 10^{-3}:					
a) Autoxidation controls	(6) 26.9 ± 4.8	(6) 26.5 ± 5.9	(4) 24.4 ± 10.1	(12) 24.3 ± 7.1	(8) 29.2 ± 7.8
b) Endogenous oxidation (0.3 mM PTU) per mm² cuticle	(6) 2.9 ± 0.8	(6) 4.8 ± 1.3	(3) 1.4 ± 0.2	(12) 5.8 ± 2.0	(7) 3.7 ± 1.1
c) Enzyme activity per mm² cuticle	(6) 4.6 ± 1.2	(6) 8.0 ± 2.3	(4) 9.0 ± 1.2	(12) 7.9 ± 4.0	(7) 8.6 ± 2.3

Table 2

Owygen uptakes by AYR and NCL females after allowing for autoxidation of the substrates ; 25°, pH 6.8, 0.09 M ACES, 25 mM ascorbic acid, 0.025 mM EDTA, n = number of replicates.

	$\bar{x} \pm se$ µl O_2/mm^2 cuticle/min $\times 10^{-3}$	
Population	*Endogenous oxidation (0.33 mM PTU)*	*Enzyme activity (no PTU)*
AYR	3.1 ± 0.4 ($n = 5$)	2.6 ± 0.9 ($n = 6$)
NCL	2.6 ± 0.5 ($n = 6$)	1.7 ± 1.0 ($n = 6$)

vity. The rest is due to endogenous oxidation. Factors such as pH and temperature probably affect these uptakes but were not studied. Consequently, results from all subsequent experiments in which catechol was added could not be adjusted to allow for enzyme activity on endogenous substrate.

Effect of pH (Fig. 2)

O_2 uptakes of NCL and AYR females were compared at 25°, pH 6.0 to 7.2 (the useful buffering range of ACES is 6.4-7.4) in 0.09 M ACES, 1mM catechol, 25 mM ascorbic acid, 0.025 mM EDTA and, if present, 0.33 mM PTU. Over this range, pH had little effect on either endogenous oxidation or enzyme activity (Fig. 2 B, C). Increased oxidation by the controls (Fig. 2 A) was expected as catechol undergoes increased oxidation as pH increases to neutrality and beyond. The only significant differences (t-test) between the two populations were in their endogenous oxidation at pH 6.4 ($p < 0.01$) and 6.8 ($p < 0.05$). However, a previous comparison of endogenous oxidation at pH 6.8 (Tab. 1) revealed no significant difference. As the present comparison is near the 5 % level of rejection, it is probably statistically, but not biologically, significant. To allow for this variability, subsequent comparisons were based on rejection of the null hypothesis at $p = 0.025$.

Effect of temperature (Fig. 3)

O_2 uptakes of NCL and AYR females were compared over the temperature range 5-37°, pH 6.8, with other conditions as documented in the preceeding section. There were no significant differences between the results for the two populations ($p < 0.025$). More interesting however was that temperature had little effect on the enzyme which was, if anything,

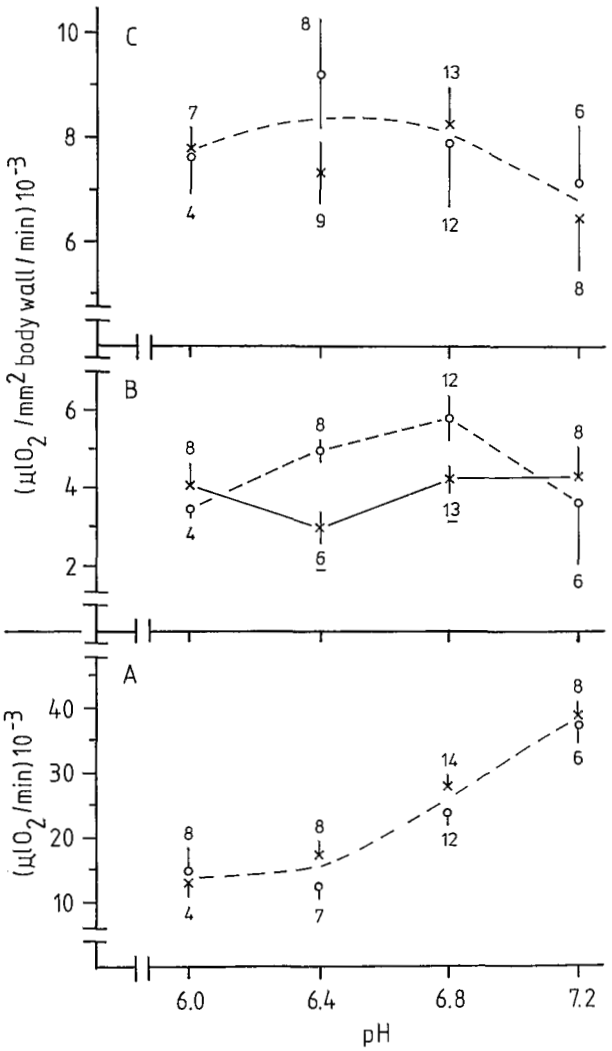

Fig. 2. Mean and standard errors for oxygen uptakes at 25° by NCL (X) and AYR (O) females in 0.09 M ACES buffer, 1 mM catechol, 25 mM ascorbic acid and 0.025 mM EDTA at different pH's. A : autoxidation controls (no nematodes) ; B : endogenous oxidation controls (females plus 0.33 mM PTU present, values for autoxidation subtracted) ; C : oxidation due to enzyme activity (values for A and B subtracted). Numbers indicate replications, underlined numbers indicate significantly different means, $p < 0.05$.

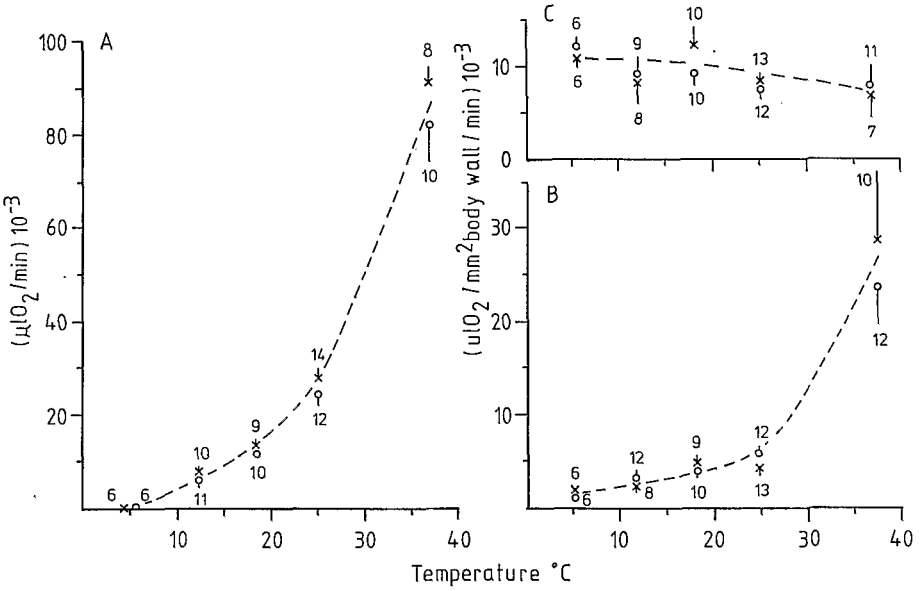

Fig. 3. Mean and standard errors for oxygen uptakes at various temperatures by NCL (X) and AYR (O) females, pH 6.8, other conditions as for Fig. 2. No significant differences between NCL and AYR females, $p < 0.025$.

more active at 5° than it was at 37° (Fig. 3 C). The means at 37° include four AYR and two NCL analyses that detected no enzyme activity ; such zero values did not occur at the other temperatures. The remarkable insensitivity of the enzyme to increasing temperatures contrasts with the typical responses shown by the autoxidation (Fig. 3 A) and endogenous oxidation (Fig. 3 B) controls. An Arrhenius plot for the latter two groups produced a good fit to a straight line.

Effect of concentration of catechol (Fig. 4)

O_2 uptakes by NCL and AYR females were measured as the concentration of catechol increased from 1 to 10 mM at pH 6.8 and 25°, with other conditions the same as those in the section on pH. The results for oxidation in the absence of catechol are those presented earlier. Significant differences ($p < 0.025$) between the mean endogenous oxidation values (Fig. 4 B) occurred at 2.5 and 10 mM catechol, with the activity from NCL females higher than that from AYR females over the range 2.5-10 mM catechol. Similarly, the curves generated by the activity of the enzyme differed (Fig. 4 C). The enzyme from the NCL females decreased in activity after a maximum at 5 mM catechol, while that from the AYR females continued to increase in activity. The mean enzyme activities differed significantly ($p < 0.025$) only at the 10mM concentration of catechol. None of the differences between the means for the autoxidation controls (Fig. 4 A) for the two types of females were significant.

Discussion

These experiments have shown that the tanning process of cyst-nematodes can be quantified by measuring oxygen uptake. Moreover, when females from two populations were analyzed, some differences were apparent. Ellenby and Smith (1967) also compared tanning of females, but they compared species rather than populations of the same species. While admitting that their arbitrary scale for measuring tanning had serious limitations for comparative purposes (they subjectively scored females from 0 for white to 5 for brown or black), they nevertheless showed distinct differences between the rates of tanning of *G. rostochiensis*, *Heterodera avenae* and *H. schachtii* at different temperatures. They speculated that the higher activity of the *G. rostochiensis*

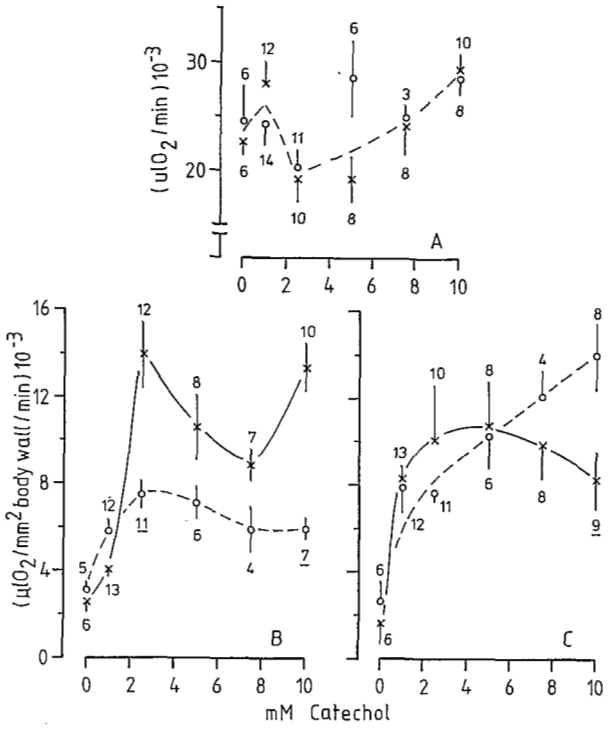

Fig. 4. Mean and standard errors for oxygen uptakes by NCL (X) and AYR (O) females at different concentrations of catechol, pH 6.8, 25°, other conditions as for Fig. 2. Underlined numbers indicate significantly different means, $p < 0.025$.

enzyme at low temperatures was correlated with the fact that this species originated in the comparatively cold environment of the high Andes of South America. The different responses of the AYR and NCL populations of *G. rostochiensis* to increasing concentrations of catechol in the present study suggest that selection pressures may be even more localized and result in populations of the same species that differ in some of their tanning characteristics. In this case, the NCL population has been cultured in the laboratory since 1941 and has been subjected to selection pressures unique to that artificial environment, while the AYR population had only recently been introduced to the laboratory when these experiments were performed. It is now known that the AYR population has adapted to selection pressures caused by continuous cultivation of early potatoes (Hominick, 1979, 1982). More extensive studies of the kinetics of the tanning enzyme of different species, pathotypes and populations of cyst-nematodes would ascertain whether any characteristics of the tanning enzyme are consistently useful for diagnostic purposes.

The Warburg method is not the best for studying kinetics of the tanning enzyme. Variability in the data necessitated several replicates, while only two autoxidation controls, two endogenous oxidation controls and two enzyme activity flasks could be assessed in one day. The method is too laborious and time-consuming. Most studies of phenoloxidases now utilize either direct spectrophotometric methods, which measure formation of the coloured quinone produced by activity of the enzyme (e.g. Gutteridge & Robb, 1975) or indirect ones, which follow the rate of disappearance of ascorbic acid (e.g. Patil & Zucker, 1965). Before they can be used however, a method for extracting the enzyme from females must be developed. An earlier study (Awan & Hominick, 1982) and results documented above suggest that the enzyme is soluble, so preparation of an active enzyme extract should be possible.

The oxygen uptake termed ,,endogenous'' is intriguing. Atkinson and Ballantyne (1977) measured oxygen consumption of *G. rostochiensis* cysts after seven days' soaking in tap water and found that for a typical cyst weighing 100 µg, about 0.05-0.06 µl O_2/h were consumed at 20°. In the present study, females, not cysts, were assayed and the temperature and medium were different. Nevertheless, the mean and standard deviation for the endogenous oxidation of the females in Table 2 were 0.29 ± 0.07 µl O_2/female/h for the AYR females and 0.18 ± 0.09 µl O_2/female/h for the NCL ones. Part of this uptake can probably be attributed to the respiration of eggs, but filtrates of crushed females still showed oxygen uptakes in the presence of PTU (Hominick, unpubl.). Also, the substantially higher uptakes by females compared to cysts may be an experimental artifact, attributed to the different temperatures and media employed for the assays. However, a more interesting possibility is that at least a portion of the higher uptake by females is real and is related to the tanning process. Since pseudocoelomic fluid promotes tanning, it may contain a phenolic substrate for the tanning enzyme or part of a biochemical system responsible for release of the substrate (Awan & Hominick, 1982). The cause of the endogenous oxidation (an oxidation that was unaffected by changes in pH from 6.0-7.2, showed an exponential response to increasing temperature, and increased markedly in the presence of catechol) and reasons why it frequently differed in the NCL compared to the AYR population, await further studies. These questions will not be resolved easily, for the females are biochemically complex and their biochemical contents change as they mature (Smith & Ellenby, 1967).

The insensitivity of the phenoloxidase activity to temperature is remarkable, for the velocity of an

enzyme catalyzed reaction usually increases with increasing temperature, attains a maximum at the so-called optimum temperature, and then decreases in activity as the heat denatures more and more of the enzyme. Ellenby and Smith (1967) showed that the phenoloxidase of *G. rostochiensis* is more active at low temperatures than the enzyme of *H. avenae* and *H. schachtii*. Their semi-quantitative assay showed that *G. rostochiensis* could even tan at 0° and that the enzyme activity was relatively unaffected over the range 0-15°. They suggested that 0-15° may lie towards the upper end of the temperature range for tanning activity, but results documented above show that enzyme activity was not significantly affected when the temperature was increased up to 37°. However, 37° may be close to the temperature of deactivation of the enzyme, for some of the replicates revealed no enzyme activity at this temperature. Alternatively, the large autoxidation and endogenous oxidation values at 37° may have obscured the comparatively small oxygen uptakes attributable to enzyme activity. In any case, the unusual response of the *G. rostochiensis* phenoloxidase to increasing temperatures warrants further quantitative comparisons with the same enzyme from other cyst-nematodes.

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