This note reports the isolation from soil of 3 bacterial strains selected by growth on cyclo(Gly-Pro) as carbon-nitrogen source. The 3 strains (DKP-1,2,3) have the respective American Type Culture Collection numbers 27965, 27966, 27967, and were classified as *Achromobacter* (DKP-1,2) or *Flavobacterium* (DKP-3). Cyclo(Gly-Pro) was made on a large scale by hydrogenolysis[6] of Z-Gly-L-Pro-NH_2, prepared as described earlier[6] except that Z-Gly-OH was coupled with H-Pro-OBzl,HCl by use of dicyclohexylcarbodiimide rather than via Z-Gly-Cl. In small-scale radioactive syntheses, H-L-Pro-[U-^{14}C]Gly-OH or H-Gly-L-[3,4-^{3}H]-Pro-OH was cyclized by heating in phenol[7]. Cells were grown aerobically at room temperature with vigorous stirring in liquid mineral media[8] with the addition of 0.2% cyclo(Gly-Pro), sterilized by filtration. For good growth (up to 300 Klett units[9]), each strain required the addition of small supplements of yeast extract (Difco), routinely added at 0.05%. At full growth we observed disappearance from the medium of cyclo(Gly-Pro) but could identify no specific product. Stock cultures were stored on trypticase soy broth (BBL) slants at 5 °C.

Utilization of radioactive cyclo(Gly-Pro), usually cyclo([U-^{14}C]Gly-Pro), by cells or extracts was assayed by spotting aliquots of the supernatant of incubation mixtures on thin-layer silica gel sheets. Development in n-propanol-methanol-water (7:2:3) for several hours separated residual cyclo(Gly-Pro) (R_f about 0.7) from more polar compounds such as H-Gly-Pro-OH and H-Pro-Gly-OH (R_f about 0.25). Dried sheets were cut into small squares which were placed directly in scintillation fluid for counting. Recovery of expected radioactivity in blank incubations (no cells or boiled cells) was close to 100%.

After growth on cyclo(Gly-Pro), washed whole-cell suspensions (50 mg wet cell weight/ml) of each strain utilized the radioactive substrate (final concentration, 4.4 µmoles/ml; 3.4×10^5 cpm/ml) at a rate of about 2–3 µmoles/ml/h. No radioactive peptide products were detected in these incubations. Most data were obtained with DKP-3. After growth of cells on H-Gly-L-Pro-OH, H-L-Pro-Gly-OH, cyclo(Gly-Gly), glucose-NH_3, or yeast extract, there was no consistent utilization of cyclo(Gly-Pro) by washed cells. In 1 experiment, apparent induction by growth on H-Gly-L-Pro-OH was observed, but could not be repeated; contamination of the open peptide substrate by spontaneous formation of the cyclic peptide on storage, is a possibility. After growth on cyclo(Gly-Pro), washed cells stored at 5 °C (1 week) or at −15 °C (4 weeks) completely lost ability to utilize the substrate.

Many attempts failed to obtain cell-free preparations of DKP-3 capable of utilizing cyclo(Gly-Pro). These included long or short periods of sonication (Branson Sonifier), extraction of acetone-dried cells, grinding with alumina, treatment in the Braun homogenizer or the French press, and exposure to lysozyme. Recombination of centrifugation fractions after various methods of cell breakage, or addition of yeast extract or boiled whole cells of DKP-3, failed to stimulate utilization of the cyclic dipeptide. More limited efforts to extract active enzyme from the other 2 cell strains, by sonication or alumina grinding, were also unsuccessful.

It is of parenthetic interest that s.c. injection of cyclo(Gly-L-[3,4-^{3}H]Pro) (5.8×10^5 cpm) in a 200-g albino rat led to 80% recovery of radioactivity in the urine (24 h), essentially all in the intact cyclic peptide, by chromatography. In contrast, injection of 6.5×10^5 cpm of H-Gly-L-[3,4-^{3}H]Pro-OH in a similar rat resulted in only 10% recovery of radioactivity in urine, of which half appeared to be $^{3}H_2O$, by distillation.

Our difficulty in obtaining active cell-free preparations from bacteria which metabolize cyclo(Gly-Pro), together with the dependence on added yeast extract for cell growth on this substrate, suggest the possibility of an unidentified cofactor requirement and/or a more complex initial reaction than hydrolysis to a free dipeptide. A rat experiment with cyclized and open-chain radioactive Gly-L-Pro was consistent with earlier data (with unlabeled compounds) indicating that a number of cyclic dipeptides are metabolically inert in mammals.

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2 P.G. Sammes, Fortschr. Chem. org. Naturstoffe *32*, 51 (1975).
3 E. Abderhalden, Biochem. Handb. *4*, 211, 282 (1911).
4 G.O. Schlütz, Hoppe-Seylers Z. physiol. Chem. *280*, 16 (1944).
5 V.D. Gröger, S. Johne and S. Härtling, Biochem. Physiol. Pfl. *166*, 33 (1974).
6 E.L. Smith and M. Bergmann, J. biol. Chem. *153*, 627 (1944).
7 K.D. Kopple and H.G. Ghazarian, J. org. Chem. *33*, 862 (1968).
8 E. Adams, J. biol. Chem. *234*, 2073 (1959).
9 R.M. Gryder and E. Adams, J. Bact. *97*, 292 (1969).

Effect of relative humidity on the free-living stages of *Strongyloides papillosus* (Rhabdiasoidea: Nematoda)

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Summary. The eggs and larvae are affected by the changes in the humidity level around them. The eggs do not develop below 87% relative humidity at 30 °C and 25 °C. At sub-developmental rel. hum. they remain viable for 30 h at 81%, but at 73% rel. hum. level they do not survive beyond 18 h. Survival of larvae in 100% rel. hum. is longer at 30 °C than at 25 °C and 35 °C, and also they exhibit a poor resistance to desiccation.

The effect of this environmental condition on the free-living stages of various species of *Strongyloides*, including *S. papillosus*, has been studied by a number of workers[2–6] in countries having a temperate climate. Among the environmental parameters that affect the biology of living organisms, rel. hum. plays a significant role in governing the rate of growth, survival, fecundity, and geographical distribution. The present communication is a report on the effect of rel. hum. on the free-living stages of *Strongyloides papillosus*, under tropical conditions.

Materials and methods. In order to get pure infection, young rabbits were infected with 8000–10,000 infective larvae of *S. papillosus*, obtained from cultures of sheep faecal pellets, by placing them on the clean-shaven abdominal skin for 1 h. The animals were kept in wire-bottomed cages separately. The desired level of rel. hum. was obtained in

Table 1. Effect of relative humidity on the development of eggs of *S. papillosus*

Temperature (°C)	Rel. hum. (%)	No. egg exposed (e.p.g.)	Pre-infective larvae Number	%	Infective larvae Number	%	Control Number	%
25	100	1067	42	3.9	56	5.2	596	55.8
	98	934	24	2.5	30	3.2	524	56.1
	79.3	1133	Nil	Nil	Nil	Nil	634	56
30	100	800	36	4.5	60	7.5	416	52
	96.5	1480	12	0.81	35	2.3	836	56.4
	92.5	1734	10	0.6	22	1.2	873	50.3
	87	2520	Nil	Nil	8	0.3	1298	51.5
	81	2520	Nil	Nil	Nil	Nil	1298	51.5

sealed desiccators (hereinafter referred to as humidity chambers) with the help of saturated salt solutions after the method of O'Brien[7].

The effect of humidity on the development of eggs into infective larvae was examined by placing thinly spread 2 g faecal cultures, with known number of ova determined by the e.p.g. count, in small petri dishes in the humidity chambers at 25 and 30 °C. The control cultures were maintained at 30 °C in wet condition. The cultures were exposed for 3 days, then taken out and baermanned for the recovery of larvae.

The experiments on the larvae were conducted by placing them on Whatman's filter paper (No. 1) in the humidity chambers. After being exposed for a specified length of time, each filter paper was quickly taken out and placed in a petri dish containing tap water. The living larvae were counted and their percentage determined.

Results. It would appear from table 1 that the percentage of infective larvae recovered from the eggs exposed in 2 g faecal cultures were 7.5%, 2.3%, 1.2% and 0.3% at 100%, 96.5%, 92.5% and 87% rel. hum., respectively, at 30 °C, while at 25 °C the larvae recovered were 5.2% and 3.2%, respectively, at 100% and 98% rel. hum. No larvae developed below 81% rel. hum. at either temperature. The yield of larvae in control cultures was never less than 50% suggesting that ova of *S. papillosus* require a wet medium for growth. The minimum rel. hum. level required for the development of eggs lies somewhere near 87% and not below it.

The pre-infective rhabditiform larvae were also recovered from the cultures, their percentage being 3.9% and 2.5% at 100% and 98% rel. hum., respectively at 25 °C, while at 30 °C it was 4.5%, 0.81%, 0.6% at 100%, 96.5% and 92.5 rel. hum., respectively. Their presence in the baermanning fluid can be assumed from the facts that no sooner were the viable eggs in contact with water than they hatched, also the ova have a somewhat different hatching range of rel. hum. than their developmental range, and within this range, hatching goes on slowly for a certain length of time until all possible hatching has taken place. The result also shows that all the eggs that hatch do not grow into mature stages. In an attempt to determine the viability of eggs at lower rel. hum. levels (81%, 73%, 31%, 10% and 4%) at 30 °C, cultures exposed for specified periods of time were taken out, moistened, and incubated at the same temperature for 84 h before baermanning.

The results in table 2 show that a small percentage of eggs survive 30 h exposure at 81% rel. hum. their survival being inversely proportional to the exposure time. The eggs do not sustain exposures beyond 18 h at a rel. hum. level of near 73% and below.

To investigate the effect of rel. hum. on the survival of infective larvae and their resistance to desiccation, fresh and active larvae, numbering about 200–300, were placed on filter papers (Whatman No. 1) and exposed to 100% rel. hum. at 25, 30 and 35 °C, and to 96.5%, 92.5%, 87% and 81% rel. hum. at 30 °C, in humidity chambers. At certain intervals, the filter papers were taken out and the larvae were collected in water. Their number was determined by direct count. The larvae in 100% rel. hum. did not survive for more than 26, 20 and 10 days at 30, 25 and 35 °C, respectively, whereas, at lower rel. hum., at 30 °C, they survived only for 4 and 2 days, respectively at 96.5% and 92.5% rel. hum. All were dead within 24 h at 87% and 81% rel. hum.

To study the effect of desiccation, a known number of infective larvae was placed on cavity-slide in a drop of water which was allowed to evaporate at room-temperature until microscopic examination revealed that only a thin film of moisture was left over them. Such slides were quickly placed in humidity chambers, taken out after a specified period of time and the larvae examined in a drop of water for 30 min to ascertain their percentage of revival.

The results recorded in table 3 indicate that only 44% larvae survive at 96.5% and 22% at 92.5% rel. hum. after 10 min of desiccation, but none survive 20 min desiccation. Their exposure for 10 min at 87%, 73% and 44% rel. hum. is

Table 2. Viability of eggs of *S. papillosus* at sub-developmental relative humidity at 30 °C

Rel. hum. (%)	Exposure time in h, with % recovery of larvae 6	12	18	24	30	36
81	78.1	44.7	40.5	15.5	3.3	Nil
73	62.0	30.2	25.9	Nil	Nil	Nil
31	33.9	29.0	14.8	Nil	Nil	Nil
10	31.2	12.5	0.6	Nil	Nil	Nil
4	23.8	10.2	0.2	Nil	Nil	Nil

Table 3. Effect of desiccation on the infective larvae

Rel. hum. (%)	Number larvae exposed	Larvae alive after 10 min desiccation Number	%	Larvae alive after 20 min desiccation Number	%
96.5	75	32	42.6	Nil	Nil
	38	17	44.7	Nil	Nil
	40	18	45.0	Nil	Nil
92.5	28	6	21.4	Nil	Nil
	35	8	22.8	Nil	Nil
	26	6	23.0	Nil	Nil
87	38	Nil	Nil	Nil	Nil
	36	Nil	Nil	Nil	Nil
	40	Nil	Nil	Nil	Nil
73	42	Nil	Nil	Nil	Nil
	26	Nil	Nil	Nil	Nil
	25	Nil	Nil	Nil	Nil
44	35	Nil	Nil	Nil	Nil
	31	Nil	Nil	Nil	Nil
	40	Nil	Nil	Nil	Nil

irreversibly lethal. The control larvae in unevaporated drop of water remain actively alive.

Discussion. The free-living eggs and larvae of *S. papillosus* are subjected to a great variety of changes in their ecology; in the field they have to face fluctuations of temperature and humidity every day. The present investigation and the observations of the effect of temperature on the free-living stages of this nematode by this author[8] indicate that both temperature and humidity act as concurrent factors. The eggs develop best at 30 °C/100% rel. hum., but below 87% rel. hum. their growth is arrested. The larvae survive for several days at 30 °C/100% rel. hum., but they all die within 24 h at a rel. hum. level of nearly 87% and below. They also die if exposed to desiccation for over 10 min. Prasad[6] observed, in Canada, that the eggs of *S. papillosus* were unable to develop at 92% rel. hum. and below, and that they did not show any preference for 'actual wetness'. The results of the present study, on the contrary, are suggestive of their preference for 'actual wetness'. Prasad's[6] finding about non-preference of the Canadian strain of the *S. papillosus* larvae for actual wetness appears to be significant if it is looked at from the point of view of adaptation of the parasite to escape being embedded in ice for several months of the year. In India and other tropical situations, their preference for actual wetness is a necessary adaptation to escape desiccation and subsequent death due to prolonged periods of higher environmental temperature and dryness. Prasad's[6] statement that longevity of larvae is inversely proportional to temperature increase at a given moisture level lends additional support to the adaptibility of the larvae. It can further be argued that the survival of larvae at different rel. hum. is greatly influenced by the difference of moisture level in the air at different temperatures which bring about changes in the degree of desiccation.

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2 H.A. Kries, Am. J. Hyg. *16*, 450 (1932).
3 J.T. Lucker, U.S. Dept. Agric. tech. Bull. No. 437 (1934).
4 M.R. Reesal, Can. J. Zool. *29*, 109 (1951).
5 H. Turner and G.I. Wilson, J. Parasit. *47*, 30 (1960).
6 D. Prasad, Patna Univ. J. *20*, 15 (1965).
7 F.E.M. O'Brien, Br. Electr. allied Ind. Res. Assoc., London (1948).
8 S. Nath, Indian J. Parasit. *1*, 2 (1977).

Labeling of endotoxin lipopolysaccharide with technetium-99m pertechnetate[1]

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Summary. Bacterial endotoxin treated with technetium-99m can be visualized in living animals by gamma camera imaging.

Many attempts to determine mechanisms of endotoxin action have involved following the distribution and fate of toxin injected into experimental animals. Hexavalent ^{51}Cr is most commonly used for labeling endotoxin for subsequent identification in host tissues[4-7], but other isotopes such as tritium[8] and ^{32}P phosphate[9] as well as immunological tagging methods[10] have also been used.

Scintigraphic equipment is now available which permits localization of materials labeled with certain gamma-emitting isotopes. It is possible that such a system could be used with endotoxin to obtain instant dynamic localization of the toxin at any time following injection into an animal. Technetium-99m is an ideal isotope for such studies as it emits a 140 keV gamma ray which is optimal for the detection systems now in use. In addition, this isotope is easily obtainable and, due to its short halflife (6 h), it can be used in dual-isotope studies. Therefore, we have attempted to develop a method for labeling endotoxin with technetium-99m so that its in vivo kinetics in animal models can be studied using gamma camera imaging system analysis.

Materials and methods. *Salmonella typhosa* lipopolysaccharide (Westphal extraction, Difco) was suspended (10 mg/ml) in saline and labeled with either $[^{51}CrO_4]Na_2$ (New England Nuclear) as described by Zlydasyk and Moon[11] or ^{99m}Tc. For daily use, 10–15 mCi of ^{99m}Tc pertechnetate ($^{99m}TcO_4^-$) in saline was eluted from a Tc-99m/Mo-99 generator (Mallinckrodt).

$^{99m}TcO_4^-$ was reduced in the presence of endotoxin. 1 ml of the endotoxin suspension (10 mg) was added to 1 ml of a 1.0 mM $SnCl_2$ solution. 10 mCi of $^{99m}TcO_4^-$ in 0.5 ml saline was added and the entire mixture adjusted to a pH of 4–5 with NaOH. After incubation at room temperature for 10 min, the mixture was passed through 5 ml of sephadex G-25 gel (Pharmacia) in a glass pipette. Endotoxin with ^{99m}Tc was eluted in the 3–5 ml fractions, whereas free isotope remained on the column.

In vivo distribution of ^{99m}Tc-endotoxin was determined in 250 g Sprague-Dawley rats which were anesthetized with halothane (Ayerst Laboratories) and inoculated via the femoral vein with 0.2 ml (approximately 0.5 mg endotoxin)

Table 1. Sephadex G-25 gel chromatography of several endotoxin lipopolysaccharide solutions

	Series 1 Cr-51-endotoxin (n=3)	Series 2* Tc+Sn+endotoxin (n=8)	Series 3* Tc+Sn (n=8)	Series 4* Tc+Sn+gelatin (n=3)
	–	–	–	–
1–2	0.5%	0.2%	0.1%	0.06%
2–3	30.6%	5.0%	0.1%	14.5%
3–4	51.6%	26.3%	0.2%	34.0%
4–5	11.0%	21.4%	0.1%	27.6%
5–6	2.7%	1.8%	0.1%	2.6%
6–7	1.4%	0.4%	0.1%	2.6%
7–8	0.5%	0.2%	0.1%	2.5%
8–9	–	0.1%	0.1%	2.1%
9–10	–	–	–	1.8%
Gel	1.6%	44.7%	98%	10.9%

Results are given in mean percent of activity of isotope administered to the column.