Secondly, trypanosome induced hemolysis is inhibited by the presence of 0.005 M EDTA. It is of interest that phospholipase induced swelling of mitochondria thought to be due to the release of free fatty acids, is similarly inhibited by bovine serum albumin and EDTA and stimulated by Ca^{++}[10]. When thin layer chromatograms of chloroform extracts of freshly isolated and autolysed T. congolense were compared, significant differences were observed in their lipid composition. In particular, fresh (non-hemolytic) organisms were rich in phosphatidyl-choline, while in autolyzed (hemolytic) organisms the phosphatidyl-choline had been significantly reduced while fatty acids were markedly increased. There were also some evidence to suggest that lysophosphatidyl-choline was generated on autolysis while triglycerides were slightly hydrolyzed.

In order to determine whether the hemolytic fatty acids were derived from phosphatidyl-choline through the action of phospholipase A (EC.3.1.1.4), or from triglycerides through lipase activity, assays for these enzymes were performed on both freshly isolated and autolyzed trypanosomes. Phospholipase A activity was determined by measuring the hydrolysis of 32 P labelled rat lecithin[11]. Lipase activity was estimated by measuring the hydrolysis of glycerol tri (1-^{14}C) palmitate[12]. Freshly isolated organisms contained relatively small amounts of phospholipase activity (3.1 nmoles lecithin hydrolyzed per mg protein/h). On autolysis this rose considerably, reaching in 1 case 200–300 nmoles/mg h. No significant levels of lipase activity were found in fresh or autolyzed trypanosomes. Preliminary results from further analysis of this phospholipase activity using 2(9, 10-di^{3}H) dipalmitoyl phosphatidyl-choline (Applied Science Laboratories) as substrate[12] suggested that the phospholipase was primarily A1.

We therefore suggest that the hemolysin of T. congolense consists largely of free fatty acids and some lysolecithin derived from the action of a phospholipase A on endogenous phosphatidyl choline. The activity is latent and becomes unmasked during autolysis of the organisms. While fatty acids and lysolecithin are regularly toxic in vitro, it is not yet possible to estimate their significance in trypanosome infections in animals. One reason for this is that both are rapidly esterified[13] and bound to albumin in vivo[9]. Nevertheless, we consider that the continued generation of these factors by phospholipase A, particularly under conditions of trypanosome accumulation and destruction, within an animal's microcirculation may lead to the development of degenerative changes in vascular endothelium resulting in increased permeability and platelet aggregation. Such changes are commonly observed in T. congolense infections[14]. In addition, it is possible that the interaction of these factors with erythrocyte membranes, while not causing intravascular hemolysis, may be sufficient to stimulate their clearance from the blood stream and contribute to the anemia so characteristic of this infection[14,15]. In this connection it is pertinent to note that the hemolytic activity was not generated by the non-pathogenic trypanosome, T. lewisi. This trypanosome did not generate phospholipase A activity on autolysis, and thin layer chromatography of chloroform extracts of fresh and autolyzed T. lewisi failed to demonstrate significant hydrolysis of phospholipids or accumulation of free fatty acids.

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Selective inhibition of reproduction in aminopterin-treated nematodes

J. R. Vanfleteren and H. Avau

Laboratoria voor Morfologie en Systematiek der Dieren, State University of Ghent, Ledeganckstraat 35, B–9000 Ghent (Belgium), 4 October 1976

Summary. Aminopterin was applied to the free-living nematode Caenorhabditis briggsae and subsequent growth was recorded. Nematode populations, containing all developmental stages and selected juvenile stages, were exposed to the drug in both growth-promoting and non-promoting media. It is suggested that aminopterin creates a specific requirement for thymine in thymine-free medium. In otherwise growth-promoting medium, aminopterin-induced thymine deficiency will lead to progressively unbalanced growth and maturation and hence to sterility even after removal of the drug. The omission of essential amino acids from the medium during thymine starvation prevents larval growth and results in better reproduction and faster proliferation in aminopterin-free medium. The 4 juvenile stages exhibit a different response to thymine starvation created by aminopterin.

Aminopterin has been used as a potent inhibitor of reproduction in studies of nematode development and ageing[1–3]. The drug prevents gonad formation and thus disturbs maturation when supplied to newborn nematodes[2]. We now present some evidence suggesting that this effect is suppressed in some non-developing juvenile stages. This might be a valuable tool for selecting nematode strains with altered metabolism.

Materials and methods. Stock cultures of Caenorhabditis briggsae were maintained axenically as previously reported[4]. Gistex medium is a chemically non-defined medium that was developed originally for the large scale cultivation of Caenorhabditis elegans[5]. It supports very fast and profuse growth with C. briggsae as well. MEM is defined here as the chemically defined medium CbMM[6] (available from Gibco bio-cult, Paisley, Scotland) lacking the non-essential amino acids alanine, aspartic acid, cysteine, glutamate, glutamine, glycine, proline serine and tyrosine. These are substituted by equimolar amounts of acetate, citrate, pyruvate and glucose[7]. MEM further contains no thymine; sterols and acid precipitated haemin are included at 50 µg/ml[8]. This medium contains all

nutrients necessary for nematode growth and reproduction, but it is a poor medium that will not yield dense populations. MM is only different from MEM in that it lacks all amino acids and the substituting mixture as well. It is suitable for maintaining the nematodes alive but not for sustaining growth. The balanced salt solution used for washing nematodes is the salt component of CbMM.

Aminopterin was dissolved in 0.05 N sodium hydroxide at 1 mg/ml and sterilized by membrane filtration. It was stored in a freezer until use. Then it was applied at a final concentration of 50 µg/ml, that is approximately 10^{-4} M. All test cultures were at room temperature. Nematodes were counted as previously reported[4]. Aseptic precautions were maintained throughout all experiments. Setting up individual experiments is more appropriately presented under results and discussion.

Results and discussion. The ability of C. briggsae to resume growth after exposure to aminopterin is shown in figure 1. Unselected nematodes from a population that had been grown in gistex medium were washed thoroughly in balanced salt solution and suspended in aminopterin containing MEM for 4, 6 and 8 days. Mortality due to aminopterin was estimated as 1–2% after 4 days, increasing to 5–10% after 8 days of exposure. The nematodes were then washed 3 times with balanced salt solution and suspended again in gistex medium. Controls, taken from the original population, were washed and transferred directly to new gistex medium. The results of this experiment clearly demonstrate that aminopterin-treated nematodes resume growth with a marked lag period, which increases with increasing duration of exposure to aminopterin.

It is shown in figure 2 that this lag period is created more by exposure to aminopterin than by some inhibitory effect of delayed nematode growth per se. Nematodes, grown in gistex medium, were washed 3 times and suspended in MM with and without aminopterin for periods of 4, 6 and 8 days. After this period of delayed growth, all test cultures were washed 3 times and transferred into gistex medium. The population growth curves of nematodes suspended in aminopterin-free MM for 4, 6 or 8 days prior to incubation in gistex medium were indistinguishable from each other. The counts were pooled to give the curve represented in figure 2. From this figure, it is clear that the effect of maintaining nematodes in a state of suspended life is rather small in these conditions. But when the nematodes are also exposed to aminopterin, their fertility decreases progressively, evoking an increasing lag period prior to proliferation in aminopterin-free medium.

There is some evidence from the experiment represented in table 1 that it is the thymine deficiency created by exposure to aminopterin that leads to its characteristic effects. In this experiment, the effect of aminopterin on nematode population growth was recorded in MEM supplemented with glycine, thymine and thymidine. Unfortunately, a limiting medium such as MEM allows poor growth, so the results may be not fully convincing. Yet we feel that the following main conclusions can be drawn from this experiment. First, thymine and thymidine are equally suitable to overcome the thymine deficiency created by aminopterin. Second, there is no evidence for

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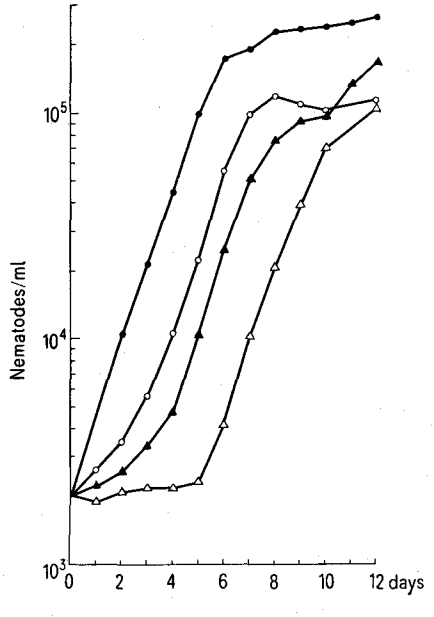

Fig. 1. Population growth of C. briggsae in gistex medium following exposure to aminopterin (50 µg/ml) in MEM. ●—● Controls not exposed to AP. ○—○ AP exposure during 4 days. ▲—▲ AP exposure during 6 days. △—△ AP exposure during 8 days.

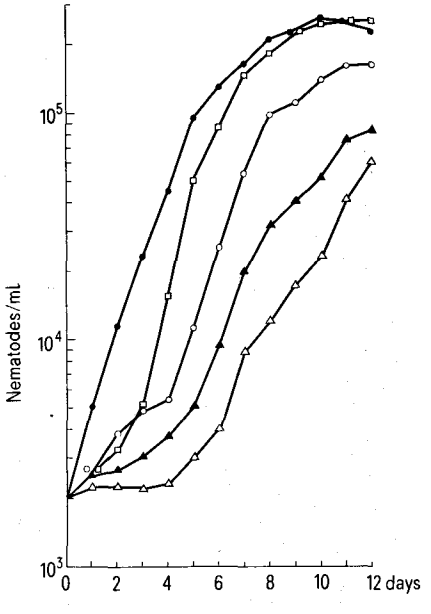

Fig. 2. Population growth of C. briggsae in gistex medium following incubation in MM with and without aminopterin (50 µg/ml). ●—● Controls not incubated in MM. □—□ Nematodes suspended in MM during 4, 6 and 8 days, pooled results. ○—○ AP exposure during 4 days. ▲—▲ AP exposure during 6 days. △—△ AP exposure during 8 days.

aminopterin-induced glycine deficiency in the presence of thymine or thymidine. This means that in nematodes, much as in HeLa cells[9], glycine must be available from sources not involving folic acid enzymes. In nematodes, such a pathway has been studied extensively[10–13]. Third, glycine alone, when supplied in large quantities is not able to overcome the growth-inhibiting effect of aminopterin, but has considerable effect in preserving the nematodes from death, i.e. it is able to reverse the direct toxic effect of aminopterin for a long period.

Like other DNA synthesis inhibitors, aminopterin selectively affects proliferating cells. Therefore it may be surprising, if not contradictory, that the addition of aminopterin to deficient media (figure 2) evokes its characteristic symptoms. More insight in this problem was gained by studying the effect of aminopterin on the various juvenile stages.

A nematode population was grown in gistex medium. Egg masses were harvested, washed thourougly in salt solution and suspended overnight in MEM. The newly hatched juveniles (J_1) were then washed and suspended for 5 days in the media shown in table 2, and allowed to reproduce in gistex medium to give the results under J_1. Other juveniles were grown further MEM until each of the 4 juvenile stages could be harvested. They were then treated as described for J_1. It may be concluded from table 2 that the first juvenile stage is most susceptible to aminopterin treatment in both media supporting (MEM) and not supporting (MM) growth. In either case, the treated J_1 did not develop any further than the second or third juvenile stage in gistex medium. In strong contrast, the second and third juvenile stages (J_2 and J_3) are very susceptible to aminopterin treatment in MEM but slightly resistant to the drug in MM. It was noticed that J_2 and J_3, treated with aminopterin in MEM, reached full length in gistex medium but did not, or only occasionally, reproduce. The fourth juvenile stage is slightly resistant to aminopterin treatment in both MEM and MM media.

The characteristic response of the 4 juvenile stages to aminopterin treatment may well be related to particularities of gonad formation during nematode development, but more experimental work would be needed to clarify further this relationship. The authors believe that it is the thymine deficiency, evoked when aminopterin is supplied to growth-promoting medium, which selectively prevents second and third stage juveniles from maturing and reproducing in aminopterin-free medium. First stage juveniles may be more affected by direct toxic effect of aminopterin. The appearance of large amounts of J_1, when unselected nematode populations are exposed to aminopterin, is responsable for the unexpected lag period in figure 2.

The findings reported here may be valuable for further study of nematode development. The selective loss of nematode reproduction when J_2 or J_3 juveniles are exposed to aminopterin in growth-promoting medium, whilst maturation and reproduction are preserved under the same conditions in deficient medium, recalls similar features of thymineless death in bacteria and cells, and may likewise be a valuable tool for isolating mutant strains that require an additional nutrient for growth.

Table 1. Inhibitory effect of aminopterin on population growth of C. briggsae and reversal by thymine and thymidine

Supplements to MEM	Population density after 14 days Expt. 1	Expt. 2
None (control	13750	14000
AP	2800	2250
AP, Gly	7950	7600
AP, Thy	11850	10200
AP, Thy, Gly	10200	10200
AP, Td	13450	10000
AP, Td, Gly	13600	9600

Supplements were added at the following molar concentrations: aminopterin 10^{-4} M, thymine 10^{-2} M, thymidine 10^{-2} M, glycine 10^{-2} M. All test media were inoculated with 2,500 nem/ml. The counts listed for MEM + AP represent living worms only. Living and dead together comprise some 6,000–7,000 due to initial proliferation during the first days of exposure to AP and subsequent increase of mortality, especially from the 7th day on. In MEM + AP + Gly less than 1% of the nematodes were dead, but worms moved very slowly. Most of the nematodes were small juveniles, which means that the population density is the result of initial proliferation during the first days of exposure. In all the other test media healty nematodes were found, predominantly adults.

Table 2. Progeny of C. briggsae juveniles treated with aminopterin *in growth promoting (MEM) and non-promoting (MM) medium*

Juvenile stage	Progeny produced after 7 and 14 days of incubation in gistex medium following pre-incubation in various media MEM (controls)	MEM + AP	MM + AP	MM
J_1	560 ± 65	0	0	200 ± 20
	16590 ± 970	0	0	11520 ± 1050
J_2	1010 ± 35	0	30 ± 10	960 ± 120
	nd	0	1280 ± 170	14780 ± 2150
$J_3 + J_2$	1400 ± 95	0	55 ± 5	920 ± 100
	nd	0 [a])	1450 ± 450	14570 ± 1180
J_3	2090 ± 100	0	55 ± 15	1080 ± 70
	19060 ± 340	0 [b])	2560 ± 450	17920 ± 1280
$J_4 + J_3$	2403 ± 22	170 ± 45	170 ± 20	1020 ± 75
	19730 ± 1440	2260 ± 700	3470 ± 210	18050 ± 390
J_4	3790 ± 930	304 ± 100	500 ± 180	2540 ± 790
	19100 ± 590	6350 ± 850	4810 ± 820	15720 ± 420

Juveniles were determined by measuring the mean length at treatment [14]. $J_2 + J_3$ means that treated juveniles were predominantly J_3 but might contain some J_2. Similarly $J_4 + J_3$ means that some J_3 may be present. Juveniles were exposed to aminopterin (50 µg/ml) or were incubated in aminopterin-free MM for 5 days and then were allowed to produce offspring in gistex medium. Juveniles, hatched and grown up in MEM, were used as controls. These were transferred immediately to gistex medium as soon as they reached the desired stage. Numbers are means ± SE of 4–8 replicate tubes. Each tube was inoculated with 5 juveniles. Variation between replicate tubes was high, especially in limiting media. [a]) except 1 tube of 8, giving a progeny of 102 nematodes; [b]) except 2 tubes of 8 which a progeny of 164 and 229 respectively; nd: not determined.

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