THE MODE OF ACTION OF THE ACYL-DIETHYLAMIDE MOLLUSCICIDES.

Thesis presented in fulfilment of the requirements for the degree of Master of Science at Rhodes University, Grahamstown.

by

Keith Ross Solomon.

DECLARATION.

I declare that this thesis is the result of my own independent investigation, except where acknowledgements have been made in the text, and that it has not been accepted for any degree, nor is it being submitted for any other degree.

Signed, K R Solomon

K. R. SOLOMON, December, 1969.

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ABBREVIATIONS

Å	Angström unit (10^{-8} cm)
ATP	Adenosine triphosphate
Ci	Curi
DEDA	Diethyldecanamide
Δt	Depression of freezing point
DELA	Diethyllauramide
DEPA	Diethylpalmitamide
DNP	Dinitrophenol
DPM	Disintegrations per minute (Refering to ^{14}C activity)
DTN	A scintillation cocktail, see Section 2.1
g	Acceleration due to gravity
nMole	One nanoMole or 10^{-9} of the molecular weight
PCP	Pentachlorophenol
ppm	Parts per million
SET	Sucrose, EDTA, Tris solution, see Section 2.1
-SH	Sulphydryl (group)
tris	Tris(hydroxymethyl)aminomethane
µMole	One microMole or 10^{-6} of the molecular weight
µM	A one microMolar solution
WHO	World Health Organization.

SECTION 1.

INTRODUCTION

That bilharziasis an important public health, social and economic problem in the tropical and subtropical countries of the world is well appreciated. (WHO, 1965) Bilharsiasis is the disease caused by parasitic worms of the genus *Schistosoma* having certain fresh water snails as the intermediate host. In South Africa the more important snails are *Bulinus africanus* and *B. globosus* , the secondary hosts of *Schistosom haematobium*. (WHO, 1965)

One method of controling the disease is to reduce the numbers of the intermediate host snails to such a low level that transmission cannot occur. (WHO, 1965 and Macdonald, 1965) This has led to a strong interest in those substances that kill snails. The first recorded use of a molluscicide, copper sulphate, was during the first world war in Egypt. (De Villiers, 1965) More recently, the more or less random testing of large numbers of compounds led to the discovery of a large number of molluscicides. These were the heavy metal compounds such as the salts of mercury and arsenic, the substituted phenols such as pentachlorophenol (PCP) and the 2-substituted 4:6-dinitrophenols. (De Villiers, 1965) These compounds are good molluscicides but suffer from a number of disadvantages. The use of copper compounds in hard waters is impractical as the copper precipitates out as the less effective carbonate. The heavy metal salts are expensive and biocidal thus making the treated water unsuitable for human or agricultural use. The phenolic compounds are unpleasant and dangerous to handle and the hydrolysis of PCP is catalysed by sunlight in clear waters. High concentrations of these compounds are required, thus increasing transport and handling costs, particularly in inaccessible areas.

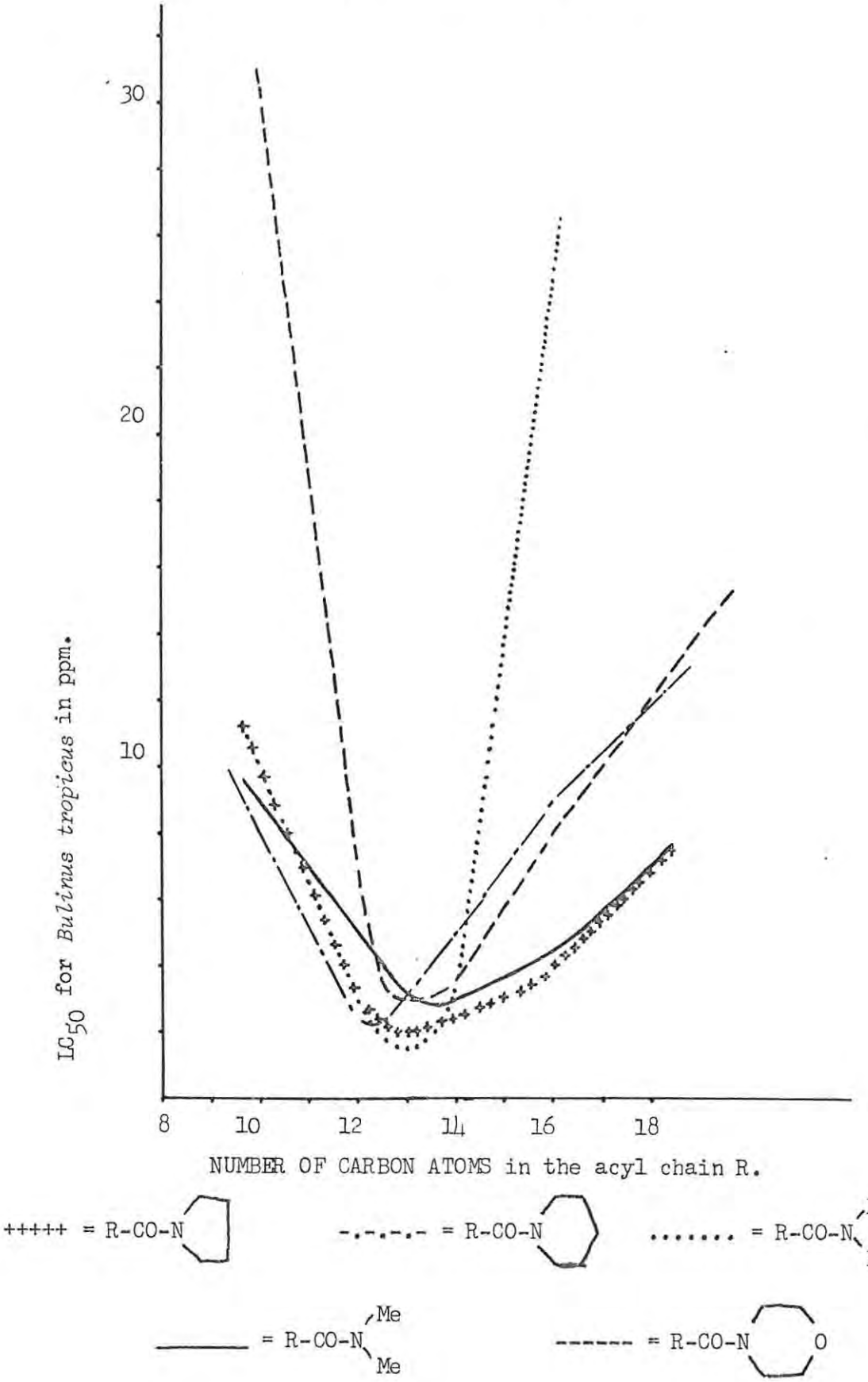
The search for an ideal molluscicide continued and recently has led to the development of compounds specifically designed as molluscicides. The systematic screening of a number of compounds shewed that 5,4'dinitrosalicylanilid had good molluscicidal properties and the subsequent variation of the substituents of this active centre produced the molluscicide, Baylucide. (2,5'chloro-4-nitrosalicylanilid)(De Villiers, 1965) Similar work at the I. C. I. and the Shell laboratories

led to the development of ICI 24233 (isobutyl-triphenyl-methylamine) and the Shell molluscicide, Frescon (triphenyl-methyl-morpholine) by Boyce *et al*(1967). These compounds are very toxic to snails and are now in commercial production.

Up until this time the trend in the development of new molluscicides has been to improve the efficacy of compounds known to have molluscicidal activity. This improvement was on a trial and error basis and there was no attempt to design a substance *de nova* which would specifically interfere with a vital biochemical process in the snails. The first known use of this type of approach was by De Villiers in this laboratory. He noted that many molluscicidal compounds such as the copper and arsenic compounds, acrolien and the organic derivitives of lead, tin and mercury are sulphydryl droup reagents. Coupling this knowledge with the fact that many lacrymators are also -SH group reagents he studied a series of lacrymatory compounds, the phenacyl halides. Using a theory which predicted the relationship between structure and lacrymatory activity (Dixon, 1948) he prepared a number of compounds of increasing molluscicidal activity, the most active of which was p-nitro-phenacylchloride with a LC_{50} of 0.9ppm for *B. tropicus* and 2.5ppm for *Tilapia mossambica* (De Villiers, 1963). This selective toxicity was attributed to the lower acid-soluble -SH content of the snails compared to other aquatic animals such as *T. mossambica*. It is thought that the acid-soluble -SH groups protect the essential -SH enzymes and that their amount is a measure of the resistance of the organism to -SH reactive compounds. (De Villiers, 1963)

It was the continuation of this line of enquiry that brought to light the molluscicidal properties of the disubstituted acyl-amides, (De Villiers, 1967) which are the subject of study in this thesis. Following up the link between lacrymatory and molluscicidal activity, De Villiers tested the effect of a lacrymator, nonanoyl morpholide, that was known to not be an -SH group reagent. It shewed no activity but the longer chain morpholides, which are not lacrymatory, shewed molluscicidal activity with maximum efficacy at a chain length of 13 carbon atoms. The testing of a mumber of structurally related compounds shewed that a number of disubstituted amides had a similar pattern of activity. (See Fig. 1, from De Villiers, 1967) Of all

Fig. 1 Variation of toxicity with acyl chain length for a number of disubstituted amides.



these compounds maximum activity is shewn by diethylmyristamide which has an LC_{50} of 1.5ppm. (De Villiers, 1967)

Although these molluscicides are not as effective as the better commercial compounds, their synthesis is simple and the starting materials are cheap and abundant. The cost of an effective dose is comparable with that of the better commercial compounds. They have a very low mammalian toxicity, (De Villiers, 1967) making them suitable for treating water subsequently used for drinking purposes. Unfortunately they are toxic to fish and experiments have shewn that the IC_{50} of diethyllauramide is the same for *T. mossambica* as for *Bulinus tropicus*.

Little work on the mode of action of the molluscicides has been reported in the literature. The copper and arsenic compounds, acrolien and the organic derivitives of lead, tin and mercury are known to poison -SH-containing enzyme systems and it is undoubtedly this property which accounts for their molluscicidal and general biocidal activity.

PCP is known to be an uncoupler of oxidation and phosphorylation *in vitro* and Weinbach and Nolan (1956) shewed that exposure of snails to this molluscicide resulted in an accumulation of acetate, lactate and pyruvate in the tissues. They suggest that this is as a result of the uncoupling activity of this molluscicide. Baylucide is also an uncoupler of oxidation and phosphorylation. (Boyce pers. comm.) According to De Villiers (1965) Baylucide and the substituted phenols interfere with osmoregulation in snails. Since the maintainance of a constant internal ionic environment is dependent on ATP as a source of energy, this effect would be expected if ATP synthesis is reduced by uncoupling of oxidation and phosphorylation. ICI 24233 also interferes with osmoregulation (De Villiers, 1965) but its effect on oxidative phosphorylation has not been reported.

Nothing is known of the biochemical action of Frescon in snails. The little work that has been done on the biological activity of Frescon has been on mammals, fish and plants and has been concerned with its detoxification and excretion. (Brown *et al* 1967; Benyon and Wright, 1967 and Griffiths, 1968) From similarities in the structure and activity between Frescon and other compounds, it appears that the toxic effect is associated with the ability of the triphenyl group

to attach itself to some nucleophilic group in the snail. (Boyce, 1965) The identity of the nucleophilic group in the snail and how it affects the organism has not been investigated.

The ultimate aim of the molluscicide research here at the National Chemical Research Laboratory is to design a molecule that will possess certain properties which cause it to interfere with a vital process specific to the snail. (Allanson, De Villiers, Schwartz and myself) One approach to this work is to make a study of the comparative biochemistry of the bilharzia vector snails and other aquatic animals, to look for differences which could be exploited to the disadvantage of the snails. However, this approach is a long term project. An alternative and possibly shorter method is to study the modes of action of known molluscicides in snails and other species to see what determines the effectivness of these compounds in different organisms, again with a view to discriminating against the snail. This was the philosophy behind the present study. The disubstituted acyl-amides were chosen because work by De Villiers shewd that moribund snails could be revived by placing them in fresh water. This suggested a purely physical mechanism of action which placed these molluscicides in a different class from all others. It was hoped that further work would open up a new field for molluscicidal research.

The work in this thesis follows on work done by A. M. Stapleberg, my predecessor at the National Chemical Research Laboratory, and some of her results are refered to in the text. Her work is, as yet, unpublished. The general approach in this work has been on two lines. Firstly the gross physiological effects of the molluscicides were studied, then followed up at a biochemical level in an attempt to explain the physiological effects. Secondly, the uptake of the molluscicides by snails was studied in an attempt to explain the differences in toxicity of these molluscicides. Much use was made of ^{14}C-labelled molluscicides for the latter work and these substances were synthesised in this laboratory. The advantage of using these tracers is that their radioactivity enables the detection of much smaller amounts of molluscicide than would be possible with standard analytical methods.

SECTION 2.

REAGENTS, GENERAL METHODS AND APPARATUS.

2.1 Solutions, reagents and chemicals.

The common laboratory reagents used in this work were of the highest obtainable purity and have not been included in this list.

Isolation of Mitochondria.

Isotonic sodium chloride.	0.9% sodium chloride in distilled water with the pH adjusted to 7 with KOH. Kept refrigerated.
SET solution.	171 g sucrose and 0.744g disodium EDTA in about 1.5L distilled water. The pH was adjusted to 7 with KOH then 100ml of 0.2M tris-Cl buffer at pH 7 was added and the volume made up to 2L. Kept refrigerated.
0.34M sucrose.	114g sucrose in 1L distilled water, the pH adjusted to 7 with KOH. Kept refrigerated.
0.125 KCl.	18.6g potassium chloride and 100ml tris-Cl buffer at pH 7 in 2L distilled water. Kept refrigerated.
Ovalbumin.	Supplied by BDH.

Warburg work on mitochondria.

Standard Reaction Mixture.	0.135g $MgCl_2.6H_2O$, 0.068 g KH_2PO_4 and 0.0405g $ATP.3H_2O$ disodium salt were dissolved in 8ml distilled water. The volume was made up to 10ml after the pH had been adjusted to 7 with KOH. The solution was made up just before use as the phosphate precicipitated out on storage.

0.5M sucrose.	17.1g sucrose in 100ml distilled water, the pH adjusted to 7 with KOH. Kept refrigerated.
0.5M succinate.	13.5g disodium succinate in 100ml distilled water and the pH adjusted to 7 with NaOH. Kept refrigerated.
10% TCA	Trichloracetic acid in water.
ATP.$3H_2O$ disodium salt and Hexokinase (Grade II)(Yeast)	were supplied by Seravac Laboratories, Cape Town.

Measurment of Radioactivity.

DTN	According to Tye and Engle (1965).
Cab-O-Sil.	Thixotropic gel powder supplied by Packard Instruments, Johannesburg.

Synthesis of Molluscicides.

Diethylamine-1-^{14}C,	with an activity of 1.14mCi/mM was supplied by Nuclear Research Chemicals, U.S.A.
Lauric acid-1-^{14}C,	with an activity of 21mCi/nM was supplied by the Radiochemical Centre, Amersham, England.
Decanoic acid, lauric acid and palmitic acid;	were all supplied by Eastman Kodak. The degree of contamination by acids of different chain length was checked by mass spectrum and found to be negligible. (Van de Walt and Eggers, pers. comm.)

2.2 Animals used in the experimental work.

The Planorbid snails, *Bulinus tropicus* were bred from an original few specimens obtained from Dr Pitchford at Nelspriut. The snails were kept in wooden trays about 200cm long, 50cm wide and 15cm deep, lined with 6000 gauge polyethylene film. The bottom of the lined tray was covered in a thin layer of gravel similar to that used in tropical fish aquaria. Pre-aerated tap water was run through the trays at a rate of about 5L per hour and the trays were kept in a room where the temperature was never allowed to drop below 18°C. Apart from the natural algal growth, the snails were fed *ad libitum* on blanched lettuce leaves every day excepting weekends.

Although *B. tropicus* is not a bilharzia vector, it is the intermediate host of *Paramphistomum microbothrium* (Seaman pers. comm.) a cattle parasite of some economic importance. *B. tropicus* were used by De Villiers (1967) for the molluscicide toxicity tests and by A. M. Stapleberg for the previous work. Because of this, *Bulinus tropicus* were used in these studies. Results obtained with this snail should be applicable to other snails.

Pyla ovata the common aquarium snail, were used for certain aspects of this work because they are large, easier to handle and more suitable for dissection than *B. tropicus*. The *P. ovata* were kept in tropical fish aquaria fitted with a heater to keep the temperature above 22°C. They were fed on blanched lettuce leaves.

The rats used in the preparation of mitochondria were from a much inbred Wistar strain. They were kept at constant temperature, humidity and day length and were fed *ad libitum* on a proprietary diet. (Epol Rat Cubes) Large (300g) male rats were taken at the same time of day (8.30am) to allow for circadian rhythm effects and were killed by cervical fracture. Excess bleeding from the nose and mouth was stopped by *post mortem* ligaturing of the neck.

2.3 Apparatus.

A Packard Tri-Carb Liquid Scintillation Spectrometer, model 574 was used for counting ^{14}C. Counting times were 10 minutes at 3°C

and the channels ratio method was used for quench correction. (Herberg, 1965)

The Warburg apparatus was of the circular type manufactured by Braun, Germany. Heating was by an inertia-less electrolytic mechanism, resulting in very stable temperatures. The Warburg manometers were of the double capillary type and were filled with Krebs manometer fluid. (Krebs, 1951) Calibrated Warburg flasks with one side-arm were also supplied by Braun and had an average volume of about 13.5ml.

A Sorval Superspeed refrigerated centrifuge was used for the preparation of mitochondria.

A Polymetron 55B pH was used for measuring pH.

Depending on the nature of the contamination, glass apparatus was cleaned in hot chromic acid, in 2% DECON in distilled water or in Tepol and hot water.

2.4 Preparation of mitochondria.

All work was done at temperatures of between 0°C and 4°C and all solutions were kept at these temperatures. The rats were killed as described in section 2.3 and the abdomen opened up the midline with two lateral cuts just below the thorax. The gut was reflected to the animals left to expose the hepatic portal vein. A hypodermic needle attached to an aspirator of cold normal saline by a length of PVC tubing was inserted into the hepatic portal vein. The posterior vena cava was cut below the right kidney and the liver perfused until almost blood-free saline was seen to flow from the posterior vena cava. The hypodermic needle was withdrawn from the hepatic portal vein and inserted into the posterior vena cava just above the right kidney. The posterior vena cava was held closed below the insertion of the needle and the liver again perfused until it took on a buff colour, indicating the absence of blood. During perfusion the liver was gently massaged with the finger to aid the removal of blood. The pressure of the saline was equivalent to a 75cm head of water.

The perfused liver was cut free from the mesentry and transfered to a cooled beaker where it was washed with SET solution and cut into small pieces with a pair of scissors. The choped liver was transfered to an all-glass Blaessig homogeniser, the volume made up to about

40ml with SET solution and the tissue homogenised with seven up and down strokes of a loose fitting pestle.

20ml 0.34M sucrose was layered under a 20ml aliquot of the liver homogenate in a cellulose nitrate centrifuge tube. This was centrifuged at 755g for ten minutes to remove the larger cell fractions. The supernatant, containing mainly mitochondria and microsomes was then centrifuged at 5090g for ten minutes to precipitate the mitochondria. Most of the supernatant was poured off and the remainder given a gentle swirl in each direction to remove the loosly packed microsomes from the mitochondrial pellet The supernatant was discarded. The mitochondria were gently redispersed in SET solution in the homogeniser and the suspension was made up to 40ml. 20ml aliquots of this suspension were centrifuged at 23500g for ten minutes, after which the mitochondrial pellet was resuspended in fresh SET solution in the homogeniser.

The protein concentration of the suspension was determined by the biuret method of Chaykin (1966) using ovalbumin as a standard. The volume of the mitochondrial suspension was then adjusted to give a protein concentration as required. The suspension was stored in crushed ice until used and was always used within three hours of preparation.

SECTION 3.

EXPERIMENTAL PROCEDURE AND RESULTS.

3.1 The effect of diethyllauramide on the water and salt balance of *Bulinus tropicus*.

The acyl-diethylamide molluscicides do not contain any of the classical chemical groups associated with enzyme inhibition and De Villiers (1967) has suggested that their action is a physical process and that the site of action is a membrane or interface in the snail. The target snails live in fresh water which, osmotically speaking, is a hostile environment and it was thought that DELA may affect the permeability or the semipermeability of the cell membrane in some way, either causing solute to leak out of or water to flow in to the snail. Either process would upset the ionic balance in the snail and could possibly cause death. The following two experiments were designed to determine if this was indeed the case.

The principle of the first is that any flow of water into the snail will cause an increase in weight which can be measured. Work on other fresh water molluscs such as *Anodonta* and *Lymnaea staginalis* (Florkin, 1948 and Huf, 1934) has shewn that narcotization of the snails with diethyl ether or with barbiturates causes an increase in weight, attributed to an inflow of water. Preliminary experiments shewed that over a period of time there were large variations in the weight of *B. tropicus* which were thought to be due to variations in the amount of air in the mantle cavity. These variations were almost eliminated by replacing the air in the mantle cavity with water according to the procedure described below.

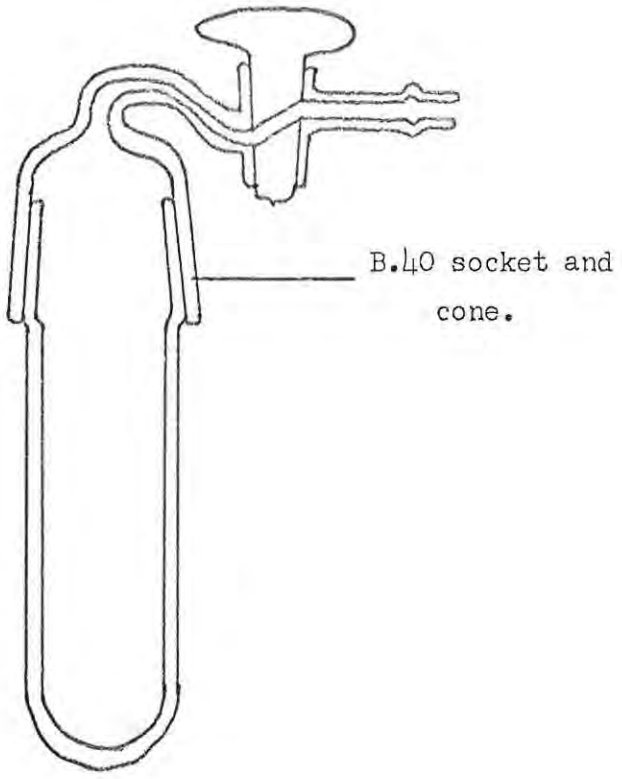
Florkin (1948) also reports that ether and barbiturates cause ions to diffuse from the haemolymph into the medium. In the second experiment the effect of DELA on the concentration of dissolved substances in the haemolymph was determined. The solute concentration of the haemolymph was measured indirectly, by the depression of freezing point.

3.1.1 The effect of diethyllauramide on the weight of *B. tropicus* .

B. tropicus were selected at random and placed in the hypobaric apparatus shewn in Fig. 2. The apparatus was evacuated to a pressure of about 7cm mercury for 30 to 40 seconds, by means of a water suction pump. At this low pressure the air in the mantle cavity expanded and most escaped. When the pressure was returned to atmospheric the air in the mantle cavity was almost totally replaced by water. The snails were held below the surface of the water by means of a stainless-steel gauze disc until weighing so as to prevent them from taking air into the mantle cavity. Before weighing, the snails were dried on a pad of tissue paper. When removed from the water the snails retracted the foot, preventing any loss of water from the mantle cavity.

Fig.2.

The apparatus used to expose *B. tropicus* to low pressure for the removal of air from the mantle cavity. The apparatus is shewn in side view and was made of pyrex glass.



The weighed snails were grouped in threes, one heavy, one light and one intermediate in weight, for easy recognition. These trios were placed in 150ml of 5ppm DELA (197ppm acetone was used to suspend the DELA in the water) in aerated tap water for certain periods of time. The DELA suspensions were prepared by adding 2.5ml of a 2mg/ml solution of DELA in acetone to one litre of tap water.

The control snails were treated in a similar manner but were exposed to 197ppm acetone only. After exposure all the snails had the air extracted from the mantle cavity, were dried and weighed as above. All work was done in a constant temeprature room at 22°C.

3.1.1 Results.

The hypobaric apparatus worked well. A visual inspection of the mantle cavity after treatment shewed that in no case was the remaining bubble of air larger than 1mm in diameter, equivalent to less than 1mg of water. The pressure did not seem to affect the snails in any way and control animals survived a 10 minute exposure with no apparent ill effects.

The weight changes of the control and experimental animals are shewn in Tables 1 to 3. In all the Tables there is a variation in the weight of the control animals before and after exposure. This is probably due to different degrees of retraction of the foot and the resulting differences in the volume and weight of water in the mantle cavity. These variations are not considered excessive.

The average weight changes in the experimental animals are not much different from those in the control and no trend is evident. This is taken as clear evidence that DELA is not causing an increase in the water permeability of those snail membranes in contact with the surrounding water.

3.1.2. The effect of DELA exposure on the depression of freezing point of *B. tropicus* haemolymph.

B. tropicus were selected at random. The experimental animals were individualy exposed to 5ppm DELA and 197ppm acetone in 100ml

(continued on page 20)

TABLE 1

Weight change of *B. tropicus* after half hour exposure to 5ppm DELA in tap water at 22°C.

Control		Experiment	
Initial weight	% Difference	Initial weight	% Difference.
169mg	-2.9%	259mg	-1.9%
160	-3.7	183	0.5
147	3.4	183	0.5
132	6.0	112	-1.8
100	3.0	76	0.0
60	-1.6	74	0.0
58	3.6	66	3.0
52	-1.9	52	-1.9
34	-2.9	52	-1.9
Average percentage difference.	-2.0		-0.5

TABLE 2

Weight change of *B. tropicus* after one and one half hour exposure to 5ppm DELA in tap water at 22°C.

Control		Experiment	
Initial weight	% Difference	Initial weight	% Difference
282mg	-7.8%	310mg	-1.9%
238	0.0	200	2.5
208	1.9	196	6.6
198	-3.0	180	2.2
163	9.2	168	7.1
161	-1.8	161	0.6
96	-6.2	108	0.8
94	1.1	102	-11.7
78	-1.3	99	1.0
44	2.3	62	4.3
33	-9.1	52	-5.7
28	-7.1	40	-2.5
Average percentage difference.	0.3		2.0

TABLE 3

Weight change of *B. tropicus* after two hour exposure to 5ppm DEIA in tap water at 22°C.

Control		Experiment	
Initial weight	% Difference	Initial weight	% Difference
170mg	-5.6%	135mg	-2.2%
137	-4.5	120	-3.3
120	0.0	118	0.0
114	-3.5	92	-1.1
107	-12.1	83	-3.6
86	-3.5	72	-1.4
83	-3.6	70	0.0
82	1.2	58	-8.6
60	1.6	54	7.4
43	-2.3	48	-2.1
42	-2.4	42	-9.5
Average percentage difference.	-2.5		-4.1

of aerated tap water in 150ml beakers. Control snails were similarly exposed to 197ppm acetone in tap water. The exposure times were 0.5, 1.5 and 3 hours, and all work was done in a constant temperature room at 22°C. After exposure the haemolymph was collected in the following manner.

The snails were removed from the beaker, dried on tissue paper, weighed, dried again and placed under liquid paraffin on a watch glass. Using a stereo microscope and fine dissection instruments the shell was broken away from round the foot and the entire foot amputated with a pair of scissors. To prevent contamination, the remains of the foot and snail were rapidly removed from the drop of blood. A sample of the blood was drawn up into a fused silica capillary tube (about 0.3mm OD) where it was isolated from the air at both ends by lengths of liquid paraffin. The silica tubes were flame sealed in labelled glass melting point tubes and stored in a thermos flask filled with solid carbon dioxide.

Before determining the freezing point, the blood was allowed to thaw and then transfered, under liquid paraffin, to a fused

silica capillary tube of smaller diameter. (about 0.1mm OD) Very small samples were taken in triplicate according to Ramsay's procedure and the freezing point determined by the method of Ramsay, *et al*, (1955). The mean Δts of the control and experimental animals were compared by Student's "t" test. Calculations were done on an Olivetti Programma 101, using the standard programme. (Olivetti 1966)

3.1.2 Results.

The results of these experiments are given in Tables 4, 5 and 6. The results in Tables 4 and 5 shew no significant difference between the haemolymph Δts of the control and experimental animals. The results in Table 6, however, shew that after three hour exposure to 5ppm DELA there is a statistically significant decrease in Δt, indicating a loss of solute from the haemolymph or an inflow of water. However, the difference between the experimental and control animals is slight and it is unlikely that this is the direct action of DELA.

TABLE 4

Depression of freezing point of *B. tropicus* haemolymph after half hour exposure to 5ppm DELA in tap water at 22°C.

	Control	Experiment
	0.305°C	0.275°C
	0.250	0.254
	0.215	0.370
	0.240	0.240
	0.245	0.235
	0.180	
	0.245	
	0.255	
Average depression of freezing point.	0.242	0.273

Student's "t" calculated for the above data is 1.230. For v = 11, $t_{0.1} = 1.796$. Therefore there is no significant difference between the two means.

TABLE 6

Depression of freezing point of *B. tropicus* haemolymph after one and one-half hour exposure to 5ppm DELA in tap water at 22°C.

	Control	Experiment
	0.265°C	0.250°C
	0.230	0.250
	0.235	0.235
	0.235	0.245
	0.270	0.235
	0.220	0.255
	0.265	0.245
	0.250	0.275
	0.230	0.260
Average depression of freezing point.	0.244	0.250

Student's "t" calculated for the above data is 0.817. For v = 16, $t_{0.1}$ = 1.746. Therefore there is no significant difference between the two means.

TABLE 6

Depression of freezing point of *B. tropicus* haemolymph after three hour exposure to 5ppm DELA in tap water at 22°C.

	Control	Experiment
	0.230°C	0.220°C
	0.255	0.240
	0.235	0.240
	0.255	0.230
	0.290	0.240
	0.250	0.240
		0.230
Average depression of freezing point.	0.253	0.232

Student's "t" calculated for the above data is 2.547. For v = 11 $t_{0.02}$ = 2.718 and $t_{0.05}$ = 2.201 . Therefore the difference between the two means is significant at the 5% level.

3.2 The uptake of the acyl-diethylamide molluscicides by *Bulinus tropicus*.

Results from the previous work (Stapleberg, pers. comm.) suggested that DELA was activly taken up by snails. It was noticed that when 20 snails were exposed to 400 ml of a DELA suspension the mortality was less than when 10 snails were exposed to a similar suspension. This suggested that the molluscicide was concentrated in the snail tissues to such an extent that the concentration in the water was reduced to a non-lethal level when too many snails were exposed together. When 10 boiled snails were exposed together with 10 live snails the mortality was the same as for 10 live snails only. This suggested that only live snails concentrated the molluscicide.

However, boiling may have denatured proteins involved in the non-active, physical adsorption of the molluscicide and this experiment was repeated. The effects of a number of metabolic poisons and methods of killing on the uptake of DELA were studied in the hope that this would indicate the mechanism of uptake of the molluscicide. As radioactive DELA was used, the uptake of the molluscicide could be measured directly.

Since previous work had shewn that there was an active concentration of molluscicide by the snails, it was considered important to know whether the uptake of the molluscicides varied with the length of the acyl chain and whether this could explain the differences in toxicity reported by De Villiers (1967). If the uptake of the molluscicides is dependent on the length of the acyl chain then the differences in toxicity would be due to differences in the uptake of the molluscicides. If, however, the molluscicides are all taken up to the same extent (when dosed at the same concentration) then the differences in toxicity would be due to differing efficacy inside the snail tissues. To study the uptake of molluscicides of different acyl chain length, ^{14}C-DEDA, DELA and DEPA were used.

3.2.1 The effects of certain metabolic poisons and methods of killing on ^{14}C-DELA uptake by *Bulinus tropicus*.

Snails were selected at random and pre-poisoned in solutions of iodoacetic acid and DNP in tap water for certain periods of time. The pre-poisoned snails were then transfered to a suspension of ^{14}C-DELA made up in aerated tap water containing the same concentration of iodoacetic acid or DNP as the snails were pre-exposed to. As a control, live non-poisoned snails were exposed in similar conditions. After exposure the amount of radioactive molluscicide in the snails was determined as below.

To determine the effect of killing by boiling on the uptake of ^{14}C-DELA, snails were boiled in tap water for 1 minute before being exposed to a ^{14}C-DELA suspension. Live snails were used as a control and the uptake of the radioactive molluscicides was determined as below.

Snails were also killed by dehydration in methanol after which the lipids and the phospholipids were extracted in three changes of a mixture of methanol and chloroform 1:2 according to Floch (1957). After a total extraction time of three days the snails were rehydrated via methanol and exposed to ^{14}C-DELA. As before the snails were selected at random and live snails were used as a control.

In all the experiments the exposure period was overnight, and all work was done in a constant temperature room at 22°C. The ^{14}C-DELA suspensions were prepared by washing the radioactive molluscicide into 500ml aerated tap water with the aid of 2.5 (197ppm)ml of acetone. The controls were exposed to 197ppm acetone only.

After exposure the snails were removed from the suspension, dried on a pad of tissue paper, weighed and then droped into plastic counting vials (Packard) standing in liquid nitrogen. The snails were ground to a powder in the frozen state with a pre-cooled all-glass pestle and then 15ml DTN was added to the frozen powder. The snails powder was suspended in the scintillation cocktail with the aid of 0.75g Cab-O-Sil thixotropic gel powder. 0.1ml samples of the molluscicide suspension were taken with a 100µL Hamilton syringe before and after exposure. These samples were added to 15ml DTN in a plastic counting vial.

^{14}C was counted in a Packard Tri-Carb. The counts per minute data were corrected for quenching and for the ^{14}C counting effiency of the counter, all by Olivetti Programma 101. (See Appendix I for the programme used.)

3.2.1 Results.

The results of these experiments are given in Tables 7 to 11. Additional data such as the exposure time and the pre-poisoning treatment are given in each table. The data are given as DPM/mg sample. The DPM are proportional to the molar concentration of the molluscicide.

The control snails in these experiments take up ^{14}C-DELA to a concentration of about 50 times, but in some cases almost 100 times that in the water. This uptake is reduced by a factor of about 5 if the snails are pre-poisoned with iodoacetic acid (Table 7), DNP (Tables 8 and 9) or are killed by boiling (Table 10). The average molluscicide concentration in snails pre-poisoned for two hours with DNP (Table 8) is much the same as that in snails pre-poisoned for 5 hours. This indicates that the uptake of the molluscicide by pre-poisoned snails is not due to insufficient pre-poisoning. This is confirmed by the results in Table 10 where the snails were killed by boiling. The high temperatures would inactivate all enzyme systems and yet these snails also take up molluscicide. Thus the uptake of these molluscicides seems to separable into two components, the passive uptake of molluscicide which is shewn by dead and pre-poisoned snails and an apparently active component which causes a much larger accumulation of molluscicide in live snails.

Table 11 shews that snails from which the lipids and phospholipids have been extracted also take up molluscicide to much the same extent as the pre-poisoned snails or the boiled snails. This indicates that the passive uptake of the molluscicide is not due to the simple solution of the molluscicide in the lipids of the cell membrane.

3.2.2 The uptake of acyl-diethylamide molluscicides of different chain length by live and dead *B. tropicus*.

The ^{14}C-molluscicides used in these experiments were kept in doses dissolved in acetone. The relevant details of these doses are

(continued on page 28)

TABLE 7

The uptake of ^{14}C-DELA from 400ml of an approximately 4ppm suspension of the molluscicide over an 18 hour period by *B. tropicus* and *B. tropicus* prepoisoned with 0.1% iodoacetic acid for 3 hours.

	Control snails	Experimental snails
Water at 0 hours	10.72DPM/mg 11.13	10.72DPM/mg 11.85
Water at 18 hours	10.66 10.04	11.49 11.49
Snails at 18 hours	394 511 343 338 278 399	122 94 62 30 123 58
Average DPM/mg snails	377	82

TABLE 8

The uptake of ^{14}C-DELA from 200ml of an approximately 4ppm suspension of the molluscicide over a 16 hour period by *B. tropicus* and *B. tropicus* prepoisoned with 0.01% dinitrophenol for two hours.

	Control snails	Experimental snails
Water at 0 hours	11.50DPM/mg 12.42	9.79DPM/mg 8.53
Water at 16 hours	5.29 5.50	5.78 5.70
Snails at 16 hours	410 364 491 433 511 350	48 38 45 83 53 71
Average DPM/mg snail	427	56

TABLE 9

The uptake of ^{14}C-DELA from 200ml of an approximately 4ppm suspension of the molluscicide over a 17 hour period by *B. tropicus* and *B. tropicus* pre-poisoned with 0.01% dinitrophenol for five hours.

	Control snails	Experimental snails
Water at 0 hours	10.41DPM/mg 10.36	10.23DPM/mg 10.12
Water at 17 hours	6.05 6.18	8.35 8.22
Snails at 17 hours	289 358 248 261 342 281	52 54 66 60 48 69
Average DPM/mg snail	297	58

TABLE 10

The uptake of ^{14}C-DELA from 250ml of an approximately 5ppm suspension of the molluscicide over a 15 hour period by *B. tropicus* and *B. tropicus* killed by boiling in tap water for one minute.

	Control snails	Dead snails
Water at 0 hours	15.73DPM/mg 15.51	16.60DPM/mg 16.91
Water at 15 hours	9.61 9.65	10.81 10.12
Snails at 15 hours	325 385 393 371 521 352	96 144 44 106 153 169
Average DPM/mg snail	391	118

TABLE 11

The uptake of ^{14}C-DELA from 250ml of an approximately 5ppm suspension of the molluscicide over a 17 hour period by *B. tropicus* and *B. tropicus* from which the lipids and phospholipids have been extracted.

	Control snails	Extracted snails
Water at 0 hours	12.26DPM/mg 12.18	12.24DPM/mg 12.15
Water at 17 hours	7.18 7.15	9.54 9.65
Snails at 17 hours	185 245 354 285 238 211	43 76 59 57 53 75
Average DPM/mg snails	253	60

given in Table 12 below.

TABLE 12

Details of molluscicide doses

	DEDA	DELA	DEPA
Weight per dose	2.12mg	2.54mg	3.3mg
µMoles per dose	9.35	9.95	9.40
Water required to make a 10 M suspension.	935 ml	995 ml	940 ml
Specific activity.	0.545 x 10^6 DPM/µMole	0.905 x 10^6 DPM/µMole	0.412 x 10^6 DPM/µMole
or	0.00183 nMoles/DPM	0.00110 nMoles/DPM	0.00243 nMoles/DPM

The molluscicide suspensions were made up in aerated tap water as in the previous experiments. *B. tropicus* were selected at random and exposed to different concentrations of ^{14}C-DELA, DEDA and DEPA overnight in a constant temperature room at 22°C. 0.1ml samples of the suspension were taken before and after exposure, and the snails were prepared for counting as in the previous section except that the plastic counting vials were cooled in solid carbon dioxide instead of liquid nitrogen. ^{14}C counts were corrected for quenching and for counting effiency as before.

In determining the uptake of DEDA and DEPA by dead snails the animals were killed by boiling in tap water for one minute. Live snails were used as a control.

3.2.2 Results.

The results are shewn in Fig. 3 and in Tables 13 to 17. The concentration of the molluscicides is given as nMoles/mg of sample weight and not in DPM/mg as before where the specific activity of the molluscicide was not known.

Fig. 3 is compiled from the results in Tables 13, 14 and 15 and shews the uptake of the three molluscicides increasing as the concentration of the molluscicide in the water is increased. Uptake is proportional to the water concentration for DEDA and DELA but not for the highest concentration of DEPA. The results for DEPA could be caused by changes in the micelle structure at high concentrations of DEPA.

When DEDA and DELA are dosed at the same molar concentration (Tables 13 and 14) the average uptake of the molluscicides is similar. However, the LC_{50}s of these molluscicides are 11 and 4ppm respectivly. (45 and 15μM) This indicates that the difference in toxicity between the two molluscicides is not due to differing uptake but to differences in efficacy inside the snail. This is not the case for DEPA which, if dosed at the same concentration as DEDA and DELA, is not taken up to the same extent. However, if DEPA is dosed at the LC_{50} of 22ppm (67μM) as in Table 15 the molluscicide is taken up to almost the same extent as DELA dosed at slightly above the LC_{50} as in Table 14. This suggests that the lower toxicity of DEPA is due to the low uptake of

(continued on page 33)

Fig. 3 Uptake of ^{14}C-DEDA, -DELA and -DEPA by *Bulinus tropicus* at different concentrations of molluscicide.

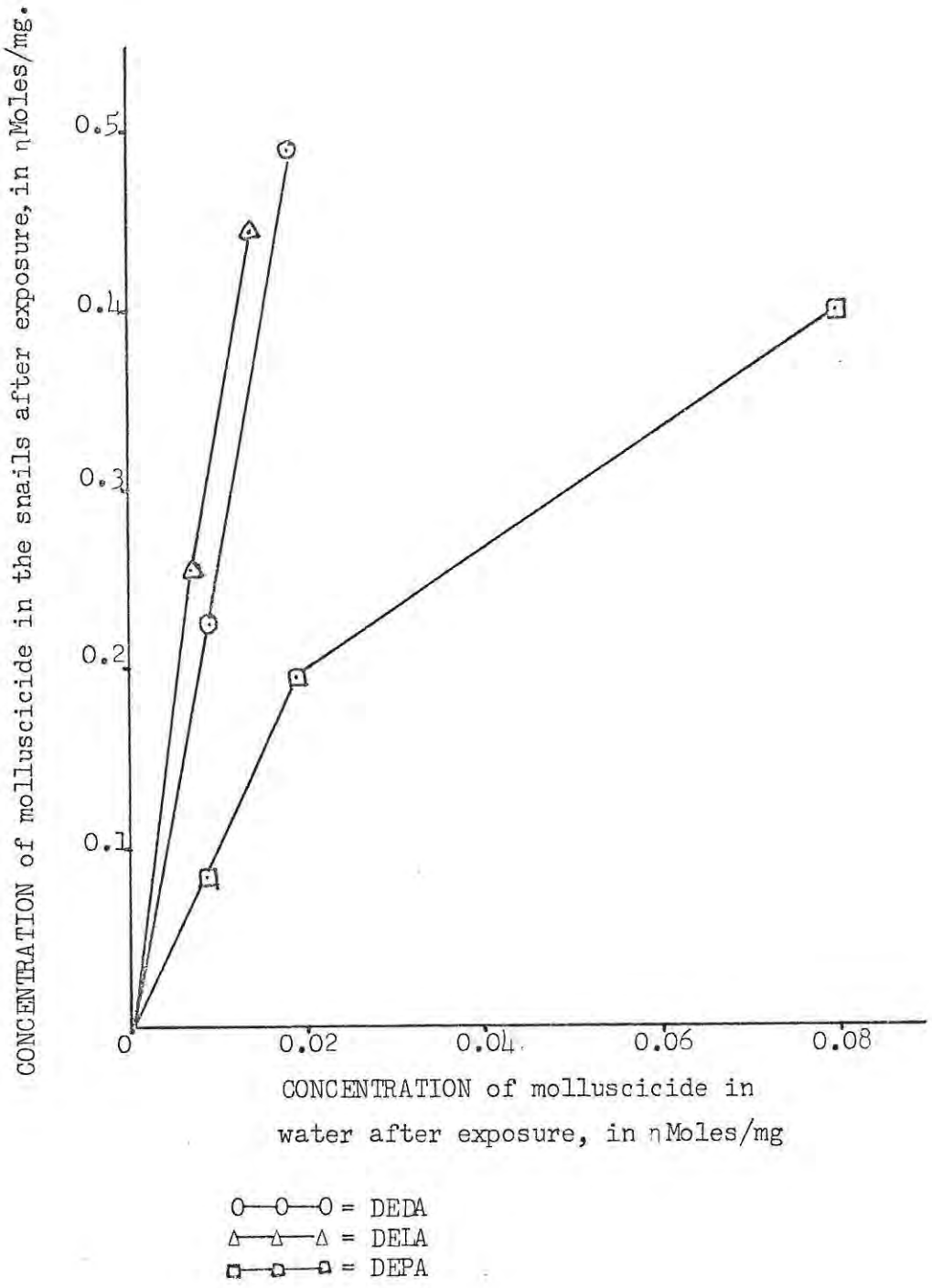


TABLE 12

The uptake of ^{14}C-labelled DEDA, DELA and DEPA by *B. tropicus* exposed to 470ml of 10μMolar suspensions for 17 hours.

	DEDA	DELA	DEPA
Water at 0 hours	0.0090nMoles/mg	0.0128nMoles/mg	0.0095nMoles/mg
	0.0089	0.0127	0.0121
Water at 17 hours	0.0088	0.0071	0.0087
	0.0088	0.0072	0.0085
Snails at 17 hours	0.045	0.129	0.094
	0.161	0.110	0.073
	0.205	0.249	0.093
	0.382	0.330	0.066
	0.197	0.345	0.089
	0.284	0.339	0.069
	0.237	0.254	0.093
	0.257	0.270	0.064
	0.236		0.057
	0.237		0.117
Average concentration in the snails.	0.224	0.253	0.082

TABLE 13

The uptake of ^{14}C-labelled DEDA, DELA and DEPA by *B. tropicus* exposed to 470ml of 20μMolar suspensions for 17 hours.

	DEDA	DELA	DEPA
Water at 0 hours	0.0197nMoles/mg	0.0185nMoles/mg	0.0217nMoles/mg
	0.0197	0.0183	0.0217
Water at 17 hours	0.0185	0.0146	0.0191
	0.0184	0.0144	0.0190
Snails at 17 hours	0.528	0.381	0.152
	0.273	0.390	0.184
	0.521	0.371	0.159
	0.497	0.429	0.214
	0.421	0.523	0.212
	0.545	0.322	0.200
	0.530	0.640	0.185
	0.615	0.517	0.244
Average concentration in the snails.	0.491	0.446	0.194

TABLE 14

The uptake of ^{14}C-DEPA by *B. tropicus* exposed to 450ml of a 67μMolar (22ppm) suspension for 17 hours.

Water at 0 hours	0.069nMoles/mg
	0.068
Water at 17 hours	0.080
	0.080
Snails at 17 hours	0.424
	0.260
	0.473
	0.540
	0.437
	0.310
	0.338
	0.377
	0.368
	0.468
Average concentration in the snails.	0.399

TABLE 15

The uptake of ^{14}C-DEDA from 492ml of a 10μMolar suspension of the molluscicide over a 17 hour period by *B. tropicus* and *B. tropicus* killed by boiling in tap water for one min.

	Control snails	Dead snails
Water at 0 hours	0.0104nMoles/mg	0.0105nMoles/mg
	0.0109	0.0105
Water at 17 hours	0.0094	0.0095
	0.0094	0.0095
Snails at 17 hours	0.113	0.033
	0.131	0.053
	0.120	0.057
	0.226	0.035
	0.210	0.056
	0.100	0.057
Average concentration in the snails.	0.149	0.048

Concentration ratio live : dead snails is 3.5:1

TABLE 16

The uptake of ^{14}C-DEPA from 500ml of a 20 µMolar suspension of the molluscicide over a 17 hour period by *B. tropicus* and *B. tropicus* killed by boiling in tap water for one minute.

	Control snails	Dead snails
Water at 0 hours	0.0225nMoles/mg 0.0225	0.0215nMoles/mg 0.0206
Water at 17 hours	0.0210 0.0211	0.0225 0.0225
Snails at 17 hours	0.133 0.118 0.124 0.155 0.170 0.264	0.038 0.040 0.054 0.059 0.039 0.082
Average concentration in the snails.	0.161	0.054

Concentration ratio live : dead snails is 3.1:1

this molluscicide by the snails and at equal concentrations inside the snail, the toxicity is the same as for DELA.

Tables 15 and 16 shew the difference in uptake of DEDA and DEPA between live and dead snails. From these results and those in Table 10, the ratios of the concentrations of the molluscicides in live and dead snails were calculated and are given in Table 17 below.

TABLE 17

Concentration ratio between live and dead snails exposed to the ^{14}C-labelled molluscicides.

Molluscicide	Ratio
DEDA	3.52 : 1
DELA	3.32 : 1
DEPA	3.10 : 1

These ratios shew that the uptake of the molluscicide by live snails is proportional to the passive uptake of the molluscicides.

In Tables 14 and 16 the concentration of DEPA in the water before exposure is lower than that after exposure. This is thought to be caused by an initial instability of the suspension resulting in increased adsorption of the molluscicide onto the walls of the syringe.. Thus the pre-exposure values are probably too low.

3.3 The effect of diethyllauramide on the respiration of *Bulinus tropicus*.

The results of the previous experiments (Section 3.1) have shewn that DELA does not have an immediate effect on salt and water balance of *B. tropicus* as was at first suspected and that the action of the molluscicide is inside the snail and is not confined to the outer membranes. This, together with the observation that the action of the molluscicide is rapid, (stress is shewn after about 10 minute exposure to 5ppm DELA) pointed to the posibility that the molluscicide had an effect on the utilization of energy by the snails. To determine if this was the case, the effects of DELA on the respiration of *B. tropicus* was investigated. Respiration was measured in the Warburg apparatus and in order to calculate the oxygen uptake it is necessary to know the exact volume of the contents of the flask which includes the volume of the snail which for the sake of calculation is regarded as part of the fluid. As it is impractical to measure the volume of the snails by individual volumetric methods, a way of determining the volume from a knowledge of the density and weight of the snails was sought.

3.3.1 The determination of the density of *B. tropicus*.

Approximately twenty snails were placed in the hypobaric apparatus as illustrated in Fig. 1. The air in the mantle cavities was extracted and replaced with water as before (Section 3.1) and the

snails were removed from the water, dried on tissue paper and weighed. The weighed snails were transfered to a 10ml measuring cylinder containing 5ml water and their volume found by displacement. The density of the snails was then calculated. In all, two determinations of density were done and the results are shewn in Table 18 below.

3.3.1 Results.

TABLE 18

Data used in the calculation of the density of *B. tropicus*.

Weight of snails	Volume of snails	Density
1.415g	1.3ml	1.10g/ml
1.304	1.1	1.18
	Average density	1.14g/ml
	or	0.87ml/g

The results of the density calculations speak for themselves. Although the shell of *Bulinus tropicus* is representative of about 15% of the total weight it has a high density and probably takes up about 5% of the total volume. It was felt that this error in calculating the fluid volume of the snail was allowable.

3.3.2 The effect of DELA on oxygen uptake by *Bulinus tropicus*.

The method for measuring oxygen uptake was that of Umbreit (1948). All glass to glass joints and the rim of the centre well of the Warburg flask were greased with Dow Corning silicone grease.

Four randomly selected snails had the air removed from the mantle cavity as described before (Section 3.1), were dried on tissue paper and weighed. The snails were then placed in 2.5ml aerated tap water in the Warburg flask and 0.1ml 10% KOH and a piece of filter paper (1.5 x 1.5cm) was placed in the centre well. 0.5ml of a DELA suspension in aerated tap water was placed in the side-arm of the flask. Equilibration time was six minutes and the oxygen consumption

was measured at 10 minute intervals. The flasks were shaken at a rate of 98 short strokes per minute and the temperature of the bath was set at 25°C.

After 50 minutes the DELA suspension was tipped into the main flask and the oxygen uptake measurments continued until the rate of uptake dropped to a fairly constant level, usually after about 60 min. Actual oxygen consumption was calculated according to the method of Umbreit *et al* (1948) using an Olivetti Programma 101. (See appendix I for the programme used.) Both total oxygen consumed per mg of snail weight and the rate of oxygen uptake (i.e. the amount of oxygen consumed between successive readings) were calculated.

The DELA suspensions were made up fresh just before use by diluting 1ml of a stock solution of DELA in 96% ethanol into 100ml of aerated tap water. The weights of DELA used to produce the final amounts of 0.6, 1.0 and 2.0μMoles DELA in 0.5ml water were 3.06, 5.10 and 10.20g DELA per 100ml ethanol respectivly.

3.3.2 Results.

The oxygen uptake results are shewn in graphic form in Figs 4 to 6. Similar results were obtained for replicate experiments but, because of differences between individual batches of snails, it was not possible to base the graphs on the averages for all experiments. It would be iterative to shew all the results here and for this reason the results of the replicate experiments and a number of preliminary experiments are given in Appendix II.

The points shewing the rate of oxygen uptake were calculated from the amount of oxygen taken up in the 10 minute period between successive readings. The scale is greatly expanded and thus small differences in the rate of uptake shew up as large distances on the graph. In Fgs 4, 5 and 6 there is an increase in the rate of oxygen uptake after the start of the experiment and before the addition of DELA. This is probably as a result of handling the snails.

The graphs shew (as do those in the Appendix II) that after the addition of DELA to the main flask there is a drop in the oxygen uptake. The rate of uptake decreases to a more or less steady

Fig. 4 The effect of 0.6μMoles of DELA on the oxygen uptake of four *Bulinus tropicus* exposed in 2.5 ml tap water at 25°C.

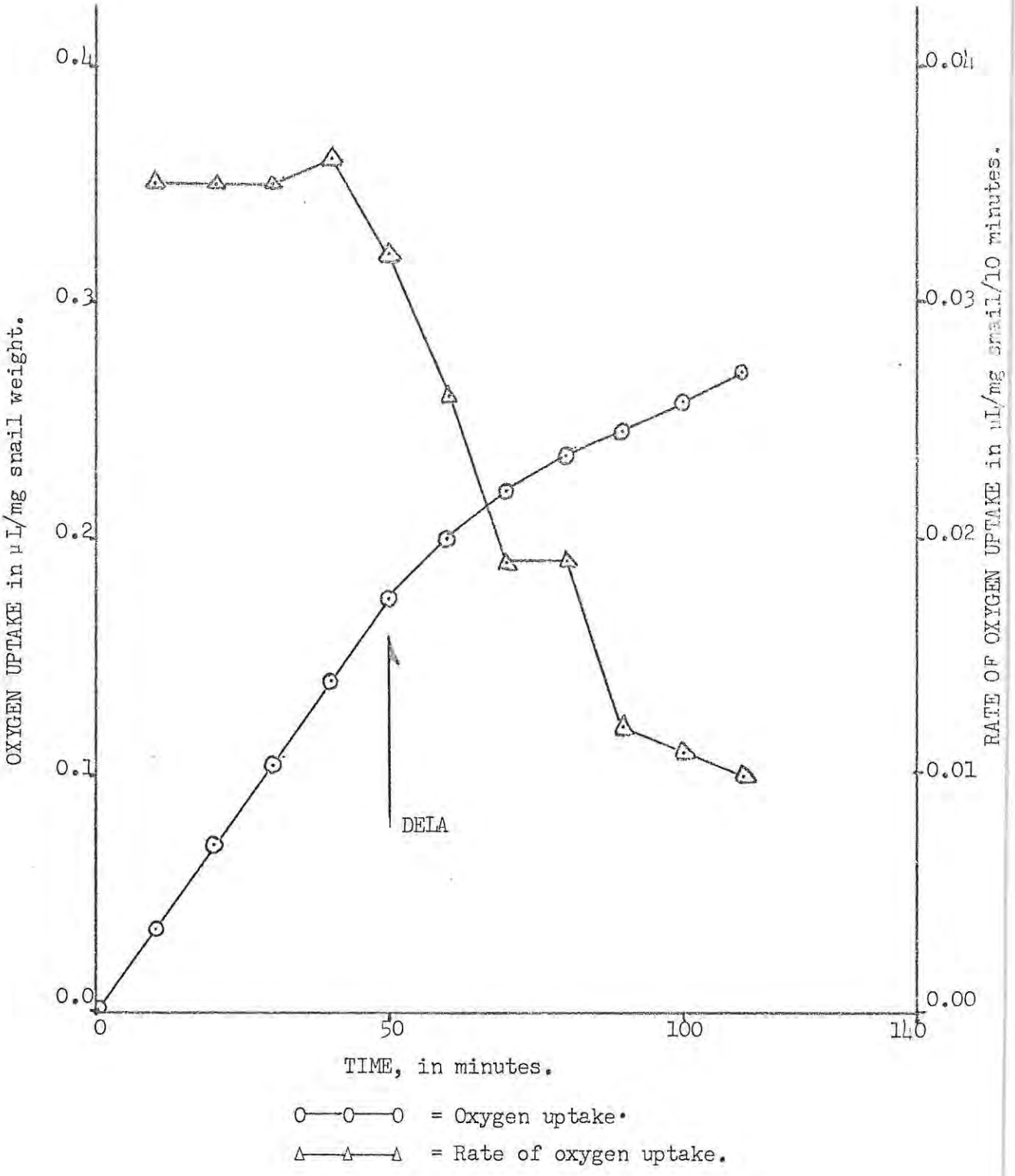


Fig. 5 The effect of 1.0 µMoles of DELA on the oxygen uptake of four *Bulinus tropicus* exposed in 2.5ml tap water at 25°C

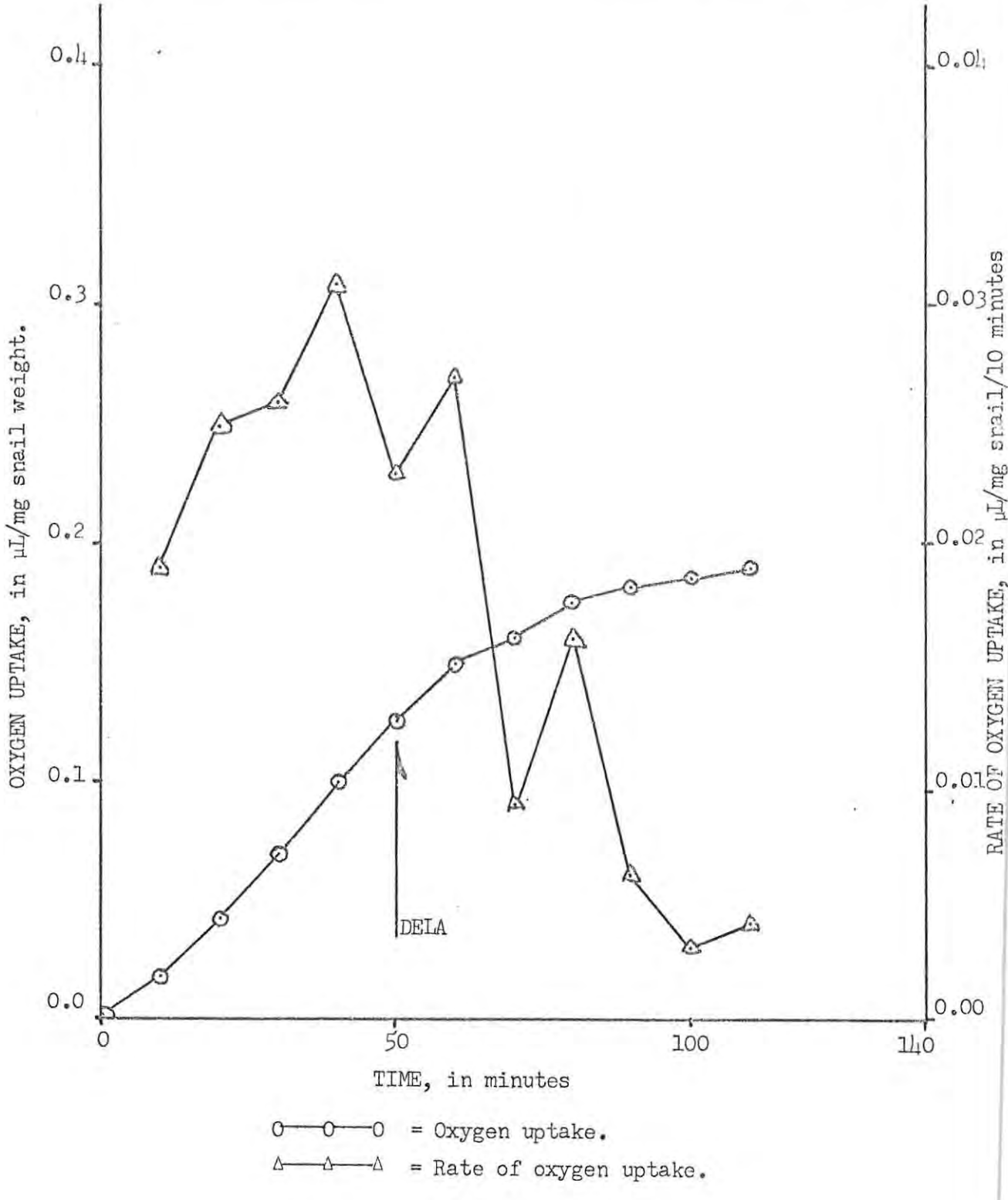
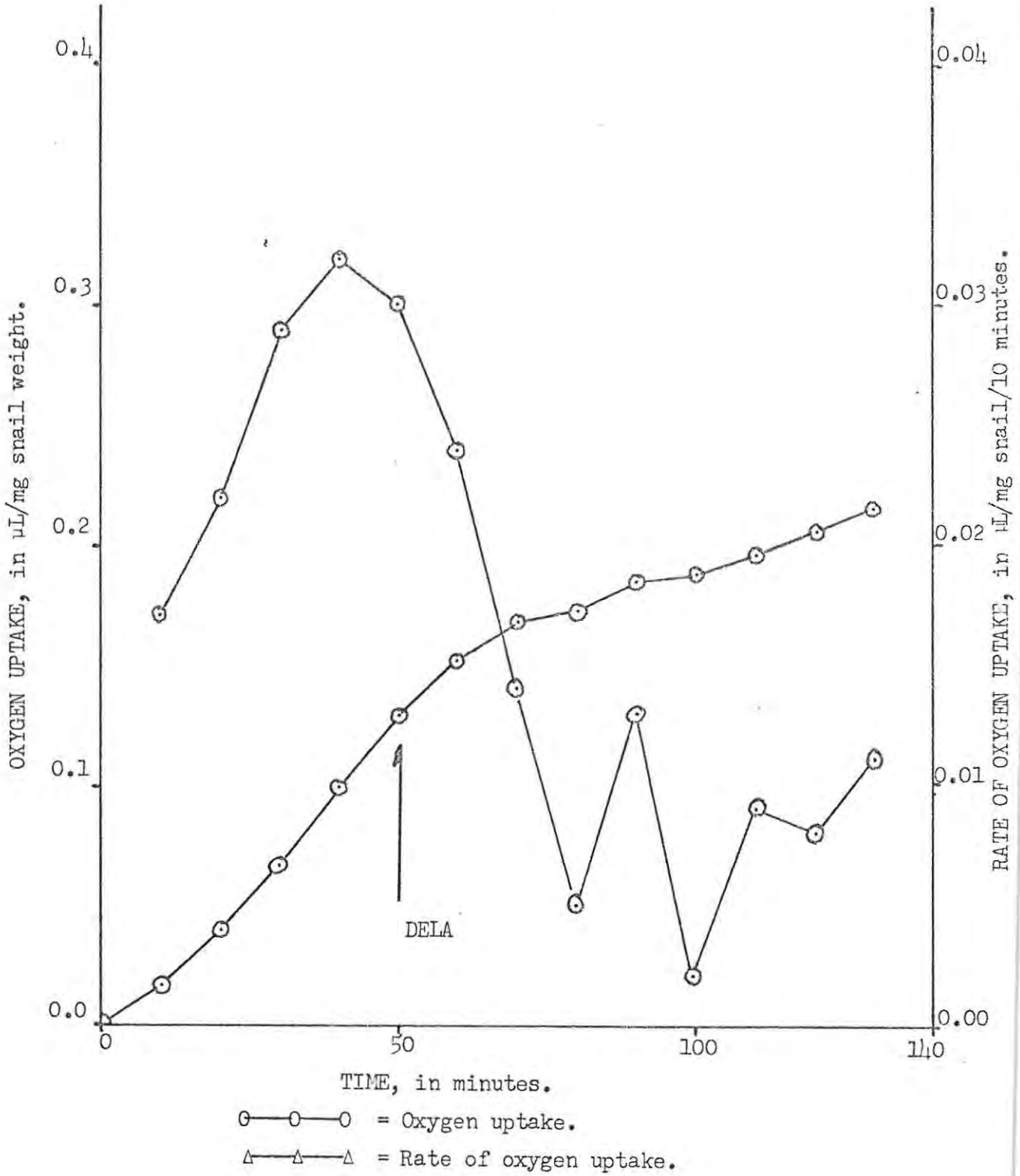


Fig. 6 The effect of 2.0µMoles of DELA on the oxygen uptake of four *Bulinus tropicus* exposed in 2.5ml tap water at 25°C.



level of about 33% of that before. This is the case for all concentrations of DELA used in the above experiments.

The snails were exposed to 0.6, 1.0 and 2.0μMoles molluscicide in 3ml water in the Warburg flask. The DELA is present in rather high concentration but this was unavoidable as larger flasks were not available. The 2.0μMoles molluscicide in the water is equivalent to 0.66ηMoles/mg, which is more than the average concentration in snails exposed to 5ppm ^{14}C-DELA for 17 hours (Table 13) where the snails took up an average of 0.45ηMoles/mg. 5ppm DELA is above the LC_{50} and therefore the 2.0μMoles DELA in the flask was more than enough to kill the snails. Visual inspection of the snails during the experiment shewed that the snails were moribund even though they were still consuming oxygen.

The reduction in oxygen uptake is not due to the DELA causing a reduction in the solubility of oxygen in the water. Preliminary experiments using an oxygen electrode have shewn that DELA has no effect on the concentration of dissolved oxygen in the water.

3.4 The effects of the acyl-diethylamide molluscicides on rat-liver mitochondria.

There is a structural similarity between the fatty acids and the acyl-diethylamide molluscicides and a number of authors have reported that the fatty acids and their derivitives are uncouplers of oxidation and phosphorylation. (Lehninger, 1964) Scholefield (1956) reported that an 0.4mM concentration of decanoate was sufficient to decrease the P:O ratio in rat-kidney and rat-brain mitochondria by one half. Tridecanoate is the most effective inhibitor of the ATP-$^{32}P_i$ exchange reaction in rat-liver mitochondria (Ahmed and Scholefield, 1960) This reaction is considered to be an essential part of oxidative phosphorylation. (Boyer *et al*, 1954)

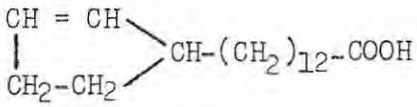
Pressman and Lardy (1956) reported that the latent "ATPase" activity of rat-liver mitochondria is stimulated by fatty acid, maximum stimulation being given by a chain length of 13 carbon atoms.

The activity of the acyl-diethylamide molluscicides varies with chain length in much the same way as is noted for the fatty acids. Quastel and Wheatly (1933) noted that the addition of fatty acid caused an initial increase, followed by a decrease in the respiration of rat-liver slices. They also noted that these effects were obtained at progressivly lower concentrations of fatty acid as the chain length increased. They found peak activity at a chain length of 12 carbon atoms. Ahmed and Scholefield (1961) studied this inhibition in rat-brain cortex slices and obtained similar results. They found that the ability of the fatty acids to produce such effects increased approximately 2.5 fold for each carbon atom added in the series from heptanoate to decanoate (7 to 10 carbon atoms). Any further increase in chain length decreased the efficacy by a similar amount for each carbon atom added.

Samson and Dahl (1955) report that the fatty acid sodium salts produce unconsciousness when given intravenously to rats. Ability to produce this effect is thought to be related to uncoupling activity and they found that the minimum effective dose decreased with an increase in chain length from propionate to decanoate. (3 to 10 carbon atoms)

Similar chain length - efficacy relationships have been reported for the microorganisms. Nieman (1954) states that "the saturated fatty acids can act as growth inhibitors, the antibacterial properties being optimal for substances of a chain length of about 12 carbon atoms." Support for the similarity in mode of action of the acyl-diethylamide molluscicides and the fatty acids comes from the work of Stanley *et al* (1932) on chaulmoorgric acid, the effective principle of chaulmoogra oil used in the treatment of tuberculosis.

Chaulmoorgric acid.



They have shewn that the carboxyl group is not essential since its replacement with the basic $CH_2\text{-}N\text{-}(CH_2\text{-}CH_3)_2$, diethylamine group results in a bactericidally active compound.

Thus the evidence seems to point to the fact that the acyl-diethylamide molluscicides are uncouplers of oxidation and phosphorylation. However, DELA causes a reduction in oxygen uptake by snails. One would not expect this if DELA were an uncoupler in the true sense of the word. A true uncoupler would only uncouple oxidation from phosphorylation and would, if anything, stimulate oxygen uptake because of removal of respiratory control. (Lehniger, 1964)

In order to clear up this rather contradictory evidence it was decided to determine the effect of the acyl-diethylamide molluscicides on a number of mitochondrial functions.

3.4.1 The effect of the acyl-diethylamide molluscicides on oxygen uptake and the coupling of phosphorylation in rat-liver mitochondria.

Initially, *Pyla ovata* hepatopancreas homogenates were tried but gave no oxygen uptake. This was thought to be due to the presence of digestive enzymes in the hepatopancreas, the site of digestion. (Owen, 1966) Attempts to isolate snail mitochondria met with no success. The animals used were *P. ovata* because of their large size and ease of handling. The sucrose method described in section 2.4 was tried on hepatopancreas and foot muscle, but with no success. The Ernster and Nordenbrand (1967) method for the isolation of skeletal muscle mitochondria was tried but again with no success. In all these cases a small pellet was isolated but this had a low oxygen uptake of about 2 µL per hour, which could have been attributed to bacterial activity. The lack of success is probably due to the paucity of mitochondria in snails.

Rat-liver mitochondria are easily prepared and can be obtained in large quantities. As mitochondria are basic to all multicellular organisms (Lehninger, 1964), results obtained with rat-liver mitochondria could point to the effects on snail mitochondria. To put it bluntly, rat mitochondria are better than no mitochondria at all.

Rat-liver mitochondria were prepared according to the procedure described in section 2.4 . The Warburg flasks were set up according to Umbreit *et al* (1948) and the following solutions were added, in the following order.

To the centre well,

0.1ml 10% KOH and
a 1.5 by 1.5cm piece of filter paper

To the side-arm,

About 1mg hexokinase powder,
0.2ml of 1% glucose.

To the main flask,

0.7ml 0.5M sucrose,
0.3ml standard reaction mixture,
0.1ml disodium succinate solution,
0.5ml mitochondrial suspension (5mg protein/ml)
0.1ml molluscicide suspension in distilled water.

The final contents of the flask were as follows,

2µMoles ATP,
15µMoles potassium dihydrogen phosphate,
20µMoles magnesium chloride,
475µMoles sucrose,
50µMoles disodium succinate,
± 1mg hexokinase and
a variable amount of molluscicide all in 1.9ml distilled water, excluding the 0.1ml 10% KOH.

As in the previous section the molluscicide suspensions were made up by diluting a stock solution of molluscicide (in 96% ethanol) into 100ml distilled water. In the case of DEPA, where higher concentrations were required, the suspension was made up in 0.5M sucrose so that a less concentrated suspension could be used. 0.5ml of the DEPA suspension was added to the Warburg flask instead of 0.1ml, and the volume of the 0.7ml 0.5M sucrose was reduced to 0.2ml so that the volumes and concentrations were the same as for the other experiments.

The molluscicides were used immediatly after making up and discarded after use because of possible adsorption on to the glassware. Similar precautions were taken when pipetting the suspensions. Rapid flow pipettes were used and the suspension was pipetted with the greatest possible haste. The pipettes were well rinsed with the suspension before use. Only in this way could good reproducibility of

results be obtained.

The Warburg flasks were set up in duplicate and as a control two flasks were set up containing everything except the molluscicide which was replaced with 0.1ml of a 1% solution of ethanol in distilled water. Two blanks were also set up containing everything except the KOH, glucose and hexokinase. The Warburg flasks were attached to their respective manometers and shaken at 112 short strokes per minute in a waterbath at 30°C. After an equilibration time of six minutes the glucose-hexokinase was tipped into the main flask. At the same time 3.0ml 10% TCA was added to the blanks followed by 0.2ml 1% glucose and about 1mg hexokinase powder. The change in pressure due to oxygen uptake was measured after a half hour. The flasks were rapidly removed from the waterbath, the filterpaper in the centre well discarded and 3.0ml TCA added to stop any reaction in the flask.

Phosphate analyses were done on 0.25ml aliquots of the TCA supernatants by Chaykin's (1966) modification of the method of Fiske and Subbarow (1925). The amount of phosphate in the experimental flasks was subtracted from that in the blanks thus giving the amount of inorganic phosphate converted into glucose-6-phosphate via oxidative phosphorylation, ATP and hexokinase. Oxygen consumption, phosphate utilization and P:O ratios were calculated with the aid of the Olivetti Programma 101. (See Appendix 1 for the programme used).

These experiments were done with varying amounts of DEDA, DELA and DEPA. The amounts of DEDA, DELA and DEPA dissolved in 100ml 96% ethanol to give a final concentration of 0.1μMoles in 0.1ml after dilution are 2.27, 2.55 and 3.10g respectively. Lower or higher concentration were made up in proportion.

3.4.1 Results.

The results are shewn in graphic form in Figs. 7 to 9. The points on which the graphs are based are the average of 4 separate determinations in the case of DEDA and DEPA and 6 determinations in the case of DELA. Relative values have been used to allow for differences from batch to batch of mitochondria. The values are relative to the

Fig. 7 The effect of increasing DEDA concentration on the P:O ratio and oxygen uptake of rat-liver mitochondria, measured over a half hour period.

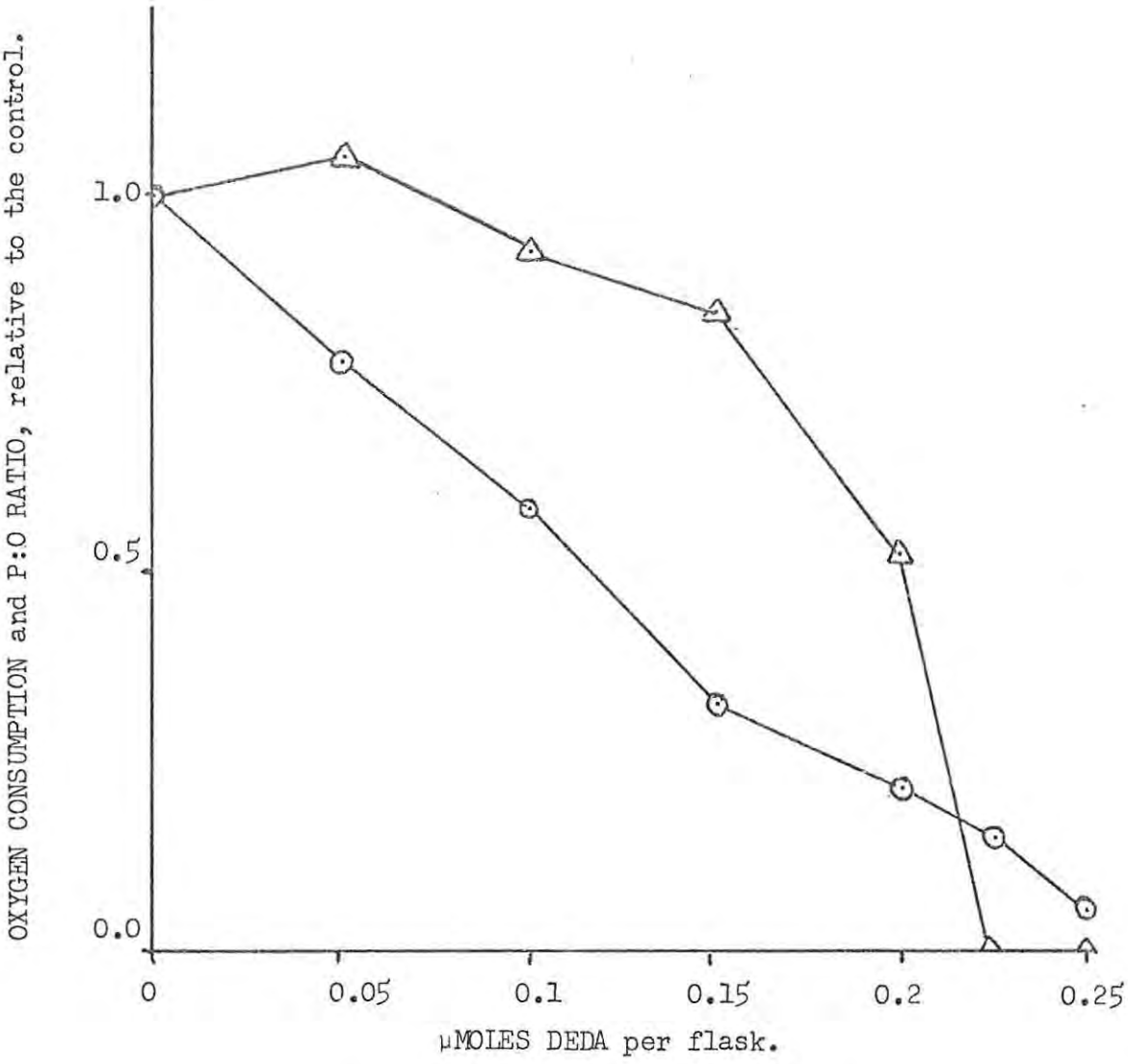


Fig. 8 The effect of increasing DELA concentration on the P:O ratio and oxygen uptake of rat-liver mitochondria, measured over a half hour period.

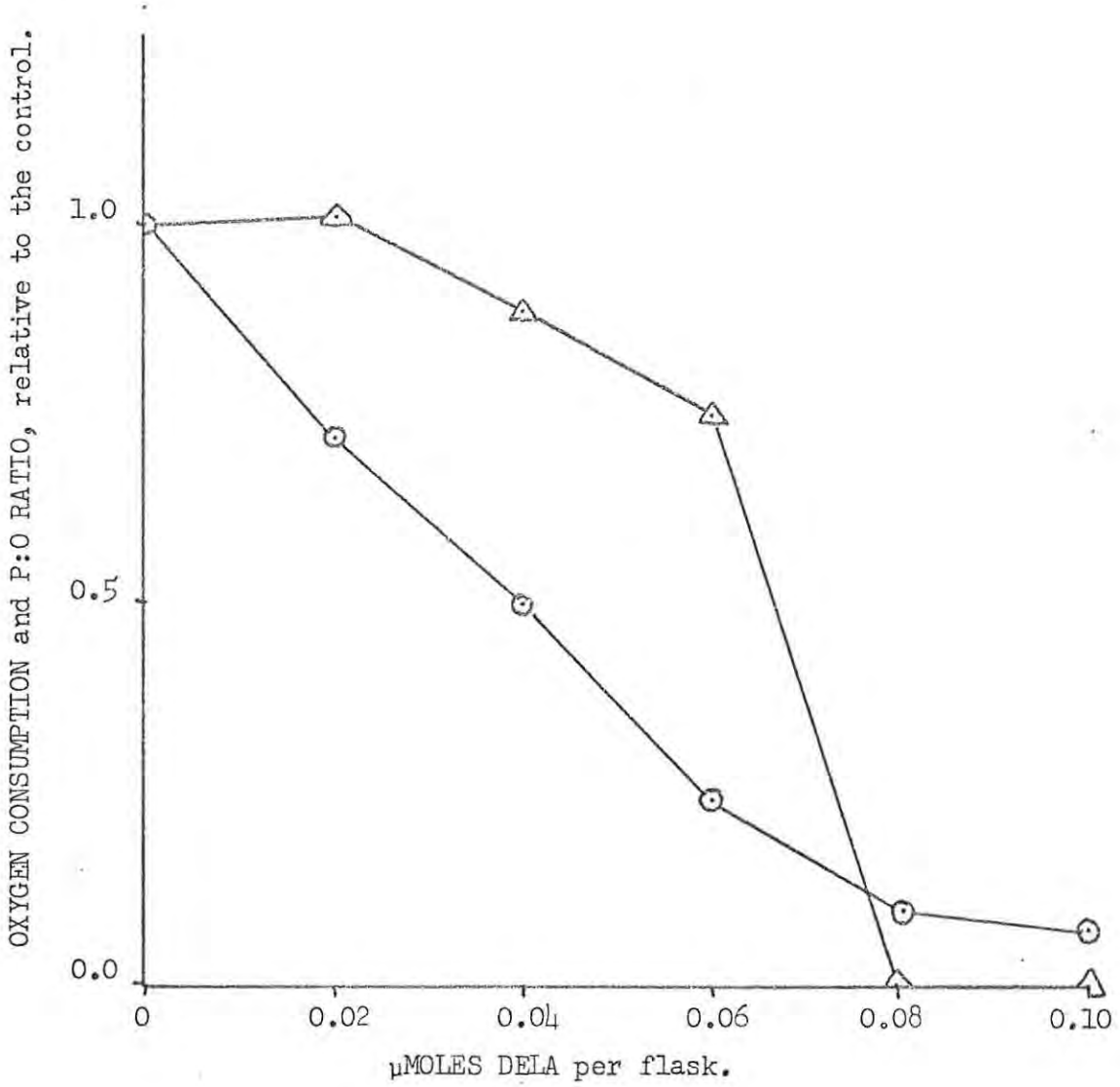
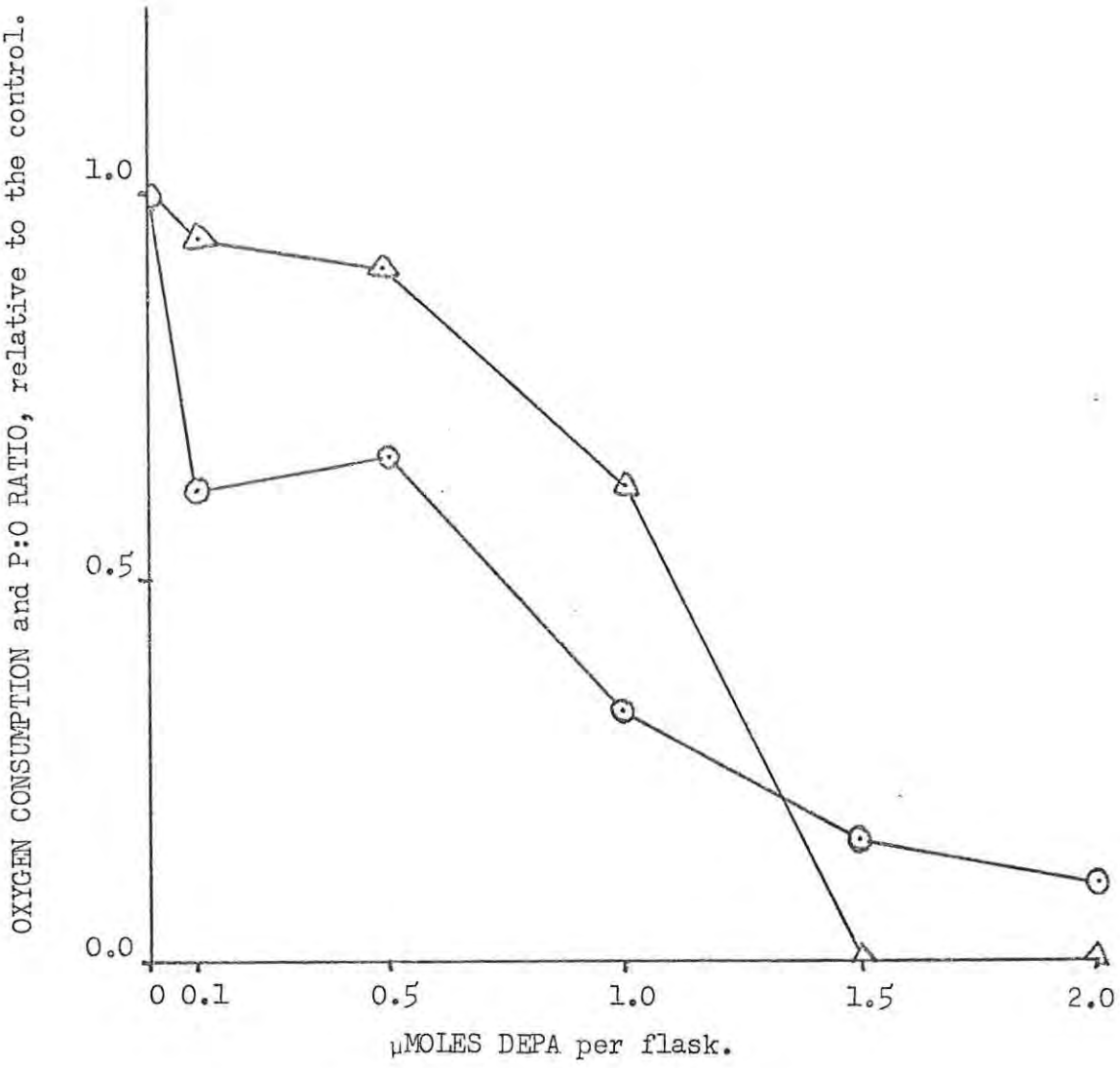


Fig. 9 The effect of increasing DEPA concentration on the P:O ratio and oxygen uptake of rat-liver mitochondria, measured over a half hour period.



control which contained ethanol in place of molluscicide and ethanol.

These results shew that for DEDA and DELA the oxygen uptake drops fairly steadily with increasing molluscicide concentration until it reaches about 30% of the control. Thereafter higher concentrations of the molluscicide cause relatively less inhibition. Also at this point there is a rapid fall in the P:O ratio which drops to zero when the oxygen consumption is at about 20% of the control. The results for DEPA are similar but there is an initial rapid decrease in oxygen consumption with little or no effect on the P:O ratio. The reason for this "step" is not known but may be due to differences in micelle properties at different concentrations of DEPA.

The effects of all three molluscicides follow much the same pattern but 2.5 times as much DEDA and about 16 times as much DEPA is required to produce the same results as DELA.

In an attempt to relate the known toxicity of the molluscicides in snails to their effects in rat-liver mitochondria, certain points on the graphs in Figs. 7,8 and 9 were compared with each other. It was hoped that only one of these ratios would be similar to the toxicity ratio, thus pointing to the precise way that the molluscicide acts in the snail. The molar LC_{50} of the molluscicides have been used as a basis for the comparison and are derived from the results of De Villiers (1967). The comparison is given in Table 20 below.

TABLE 20.

A comparison of the effects of DEDA, DELA and DEPA on the respiration of rat liver mitochondria.

	DEDA	DELA	DEPA
The LC_{50} of the molluscicides in mM.	45	15	67
Ratio of the LC_{50}s	3.0	1	4.5
µMoles molluscicide required to completely uncouple oxidation and phosphorylation.	0.225	0.08	1.5
Ratio of the above.	2.82	1	18.4
µMoles molluscicide required for 50% uncoupling of oxidation and phosphorylation.	0.20	0.067	1.10
Ratio of the above.	2.99	1	16.4
µMoles molluscicide required for 50% reduction of oxygen uptake.	0.10	0.04	0.70
Ratio of the above.	2.5	1	17.5

In the calculation of the ratios the value for DELA is taken as unity.

The ratio of the amount of DEDA to the amount of DELA required for complete uncoupling, 50% uncoupling and a 50% reduction in oxygen uptake are all much the same as the toxicity ratio of 3:1. This unfortunately gives no positive clue as to the mode of action, although the ratio for the amount of DELA and DEDA required for 50% uncoupling is almost 3:1. However, the other ratios are all close to this value and experimental error makes any choice between them very difficult.

The large amount of DEPA required to produce the same effect as DELA is unexpected as the earlier work with *B. tropicus* in section 3.2 indicated that the toxicity of DEPA inside the snail is similar to that for DELA. The reason for these unexpected results is not known.

3.4.2 The effect of DELA on swelling and ATP-induced contraction of rat-liver mitochondria.

The action of the acyl-diethylamide molluscicides on mitochondria is to reduce the oxygen uptake and to cause uncoupling of oxidation and phosphorylation. It was thought that the molluscicides may affect the integrity of the mitochondrial membrane in some way. Lehninger (1964) quotes a number of substances that cause mitochondrial swelling, amongst these the fatty acids. Swelling caused by these agents can be reversed by ATP. The study described below was undertaken to see if the acyl-diethylamide molluscicides also caused swelling.

The mitochondria were prepared as in section 2.4 except that they were suspended in 0.125M KCl instead of SET solution after the final centrifugation. (According to Lehninger, 1959) This suspension was then diluted to give a mitochondrial protein concentration of 0.25mg/ml as this concentration gave a workable optical density.

3ml of the mitochondrial suspension was pipetted into a 10mm spectrophotometer cell in duplicate. 0.1ml of a DELA suspension in 0.125M KCl was added to the one cell and 0.1ml of 1% ethanol in 0.125M KCl was added to the other. Both were well stired and the optical density at 5200Å measured against a 0.125M KCl blank. To minimise delay the DELA suspension and the ethanol solution were added to the cells in place in the spectrophotometer.

Thereafter the optical density was measured at two minute intervals until there was only a slight change between two successive readings. At this point 0.1ml distilled water containing 5mMoles ATP and 5mMoles magnesium chloride was added to the cell. The suspension was well stirred and the optical density readings continued until there was little difference between successive readings. To check that the effects after the addition of ATP were due only to ATP the experiment was repeated using only 5mMoles magnesium chloride. The DELA suspensions were made up as in the previous section.

3.4.2 Results.

The results of this experiment are shewn in graphic form in Fig. 10. DELA does not cause the expected swelling and, in fact, the DELA-exposed mitochondria swell less than the controls exposed to ethanol alone. When ATP is added, the control mitochondria shew the expected contraction but the DELA-treated mitochondria shew a rapid drop in optical density indicating rapid swelling. This effect is due to ATP as there is no rapid swelling on the addition of magnesium chloride alone.

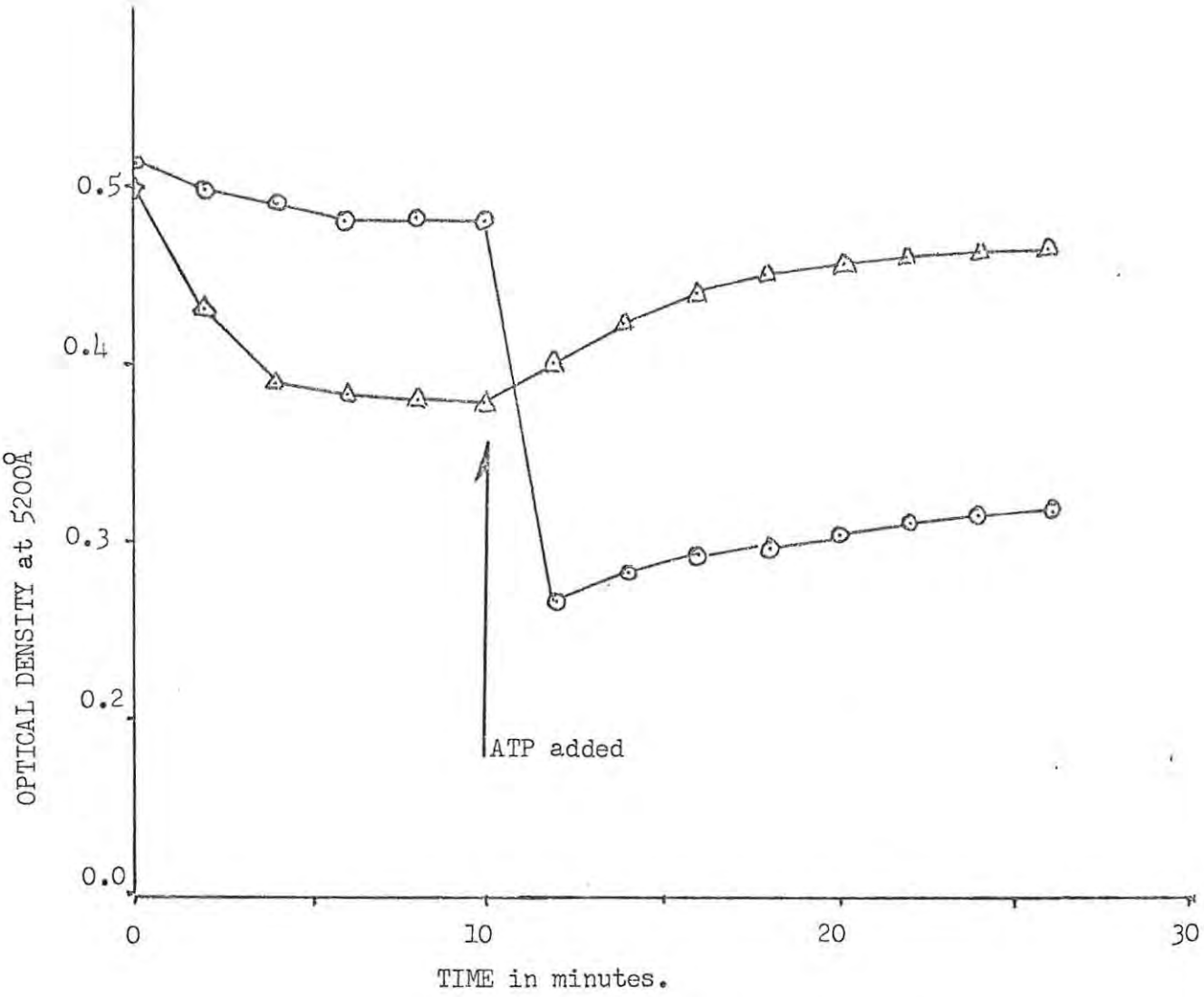
3.4.3 The effect of DELA on rat-liver mitochondrial "ATPase".

The results of the previous experiment indicated that pre-treatment with DELA causes a sudden swelling of mitochondria when ATP is added to the suspension. Lehninger (1964) states that inorganic phosphate is an active swelling agent and it was thought that the swelling in the previous experiment could have been caused by phosphate derived from the rapid hydrolysis of ATP by DELA-stimulated "ATPase".

The following experiment was designed to determine the effect of DELA on the rate of hydrolysis of ATP by mitochondrial "ATPase". The principle is that a given time after the addition of ATP to the mitochondrial suspension, a mixture of glucose and hexokinase was added to convert the unhydrolysed ATP to glucose-6-phosphate and ADP. Only the inorganic phosphate liberated by the mitochondrial "ATPase" is then determined. No Krebs cycle substrates were present thus eliminating the effect of ATP produced from phosphorylation.

The mitochondria were prepared as for the previous section

Fig. 10 The action of DELA on mitochondrial swelling and the effect of ATP on this swelling.



O O O = Control exposed to ethanol only.

Δ Δ Δ = Experiment exposed to DELA and ethanol.

and the suspension diluted to give a protein concentration of 0.25mg/ml. 4.8ml aliquots of this suspension were pipetted into test-tubes placed in a waterbath at 20°C. The temperature was allowed to equilibrate for 5 minutes after which 0.2ml of a DELA suspension in 0.125M KCI was added and well mixed by shaking. Similarly, 0.2ml of 1% ethanol in 0.125M KCI was added to the control tubes. All tubes were allowed to stand in the waterbath for a further 10 minutes.

At "zero time" 0.5ml of a 1% glucose solution containing about 2mg hexokinase was added to the first control and experimental tubes. This was followed by 0.5ml distilled water containing 5 μMoles ATP and 5μMoles magnesium chloride, added to all the test and control tubes. At intervals of 3, 6 and 9 minutes, 0.5ml glucose-hexokinase was added to the second, third and forth test and control tubes respectively. One minute after the addition of the glucose-hexokinase, 3ml of 10% trichloracetic acid was added and well mixed by shaking. 2ml aliquots of the supernatant were analysed for phosphate by the method of Chaykin (1966). The total amount of inorganic phosphate produced from the hydrolysis of ATP was calculated. The DELA suspension was prepared as in Section 3.4.1 and the final suspension contained 0.1μMoles DELA in 0.1ml of 0.125 M KCl.

3.4.3 Results.

The results are shewn in graphic form in Fig. 11. The hydrolysis of ATP, as shewn in the graph by the appearance of inorganic phosphate is not a straight line. This is not unexpected as in the above conditions the hydrolysis is dependent only on the concentration of ATP, the hydrolysis of which is a first order reaction.

Although there is stimulation of "ATPase" activity by DELA, this is insufficient to produce inorganic phosphate at the required rate to account for the rapid swelling seen in the previous experiment. It would then appear that this swelling is an active process in that it requires a source of energy.

The slopes of the hydrolysis of ATP by mitochondrial "ATPase" (Fig. 11) were calculated by the least squares method on an Olivetti Programma 101 (Olivetti, 1966) and are given in the following Table, Table 21.

TABLE 21.

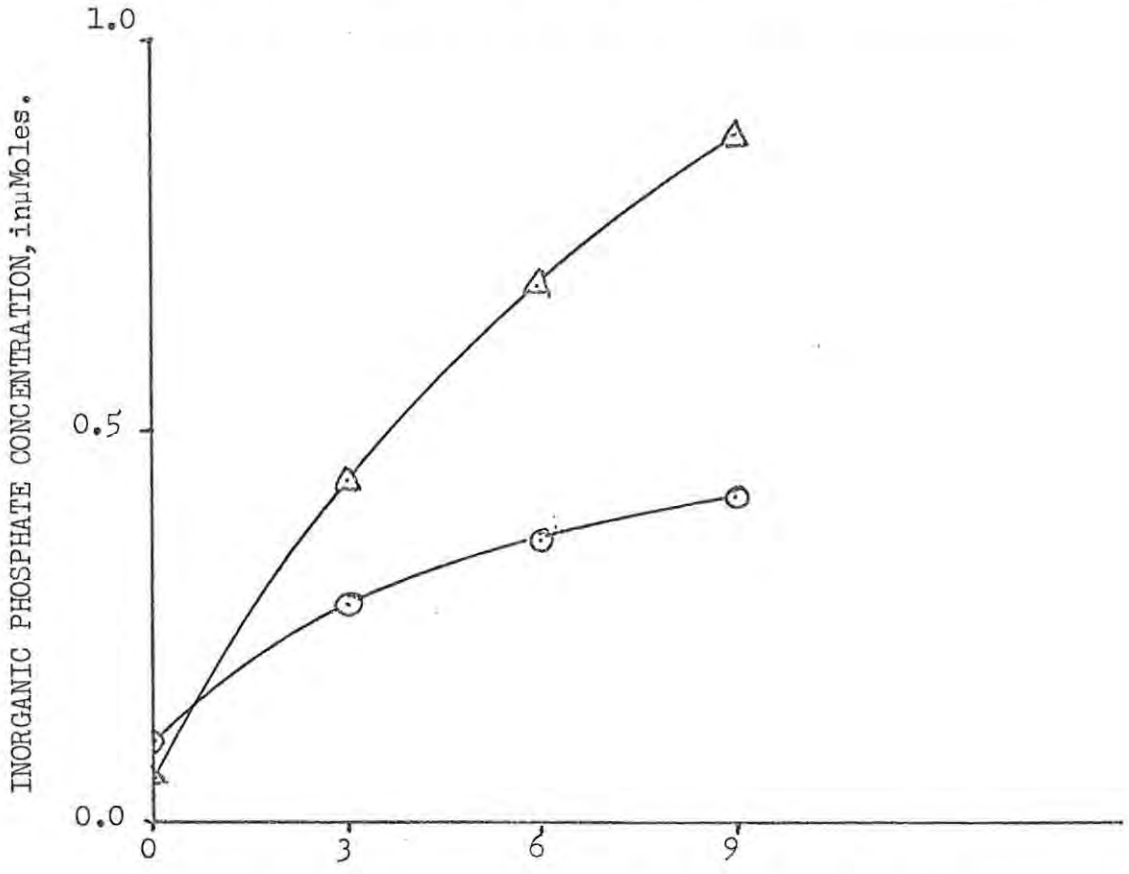
Lines of best fit for the graphs in Fig. 11.

Graph	Slope	Intercept	"r"
Ethanol control	0.023 µMoles/min	0.214 µMoles	0.997
DELA experiment	0.072 µMoles/min	0.232 µMoles	0.997

The calculations are based on the last three points only.

The rate of hydrolysis in the DELA exposed mitochondria is three times that in the control. Thus DELA stimulates mitochondrial "ATPase" activity by a factor of three.

Fig. 11 The effect of DELA on rat-liver mitochondrial "ATPase. A graph shewing the appearance of inorganic phosphate in mitochondria pre-exposed to DELA and to ethanol.



SECTION 4.

SYNTHESIS OF RADIOACTIVE MOLLUSCICIDES.

Previous to the initiation of this work the only labelled molluscicide synthesised was N,N-1-^{14}C-diethyllauramide. This synthesis was by the reaction of 1-^{14}C- diethylamine hydrochloride on lauric acid chloride in the presence of pyridine. (Holzapfel and Van de Walt, pers. comm.) The same synthetic path was used for the synthesis of ^{14}C-labelled N,N-diethyldecanamide and N,N-diethylpalmitamide. A different method was used for the synthesis of additional ^{14}C-diethyllauramide. In this case oxalyl chloride was used to produce ^{14}C-lauric acid chloride which was then reacted with excess diethylamine to form the diethyllauramide. The series of reactions is shewn below.

$$2RCOOH + (COCl)_2 \rightarrow (RCOOCO)_2 + 2HCl \quad (a)$$

$$(RCOOCO)_2 \rightarrow (RCO)_2O + CO_2 + CO \quad (b)$$

$$(RCO)_2O + (COCl)_2 \rightarrow 2RCOCl + CO_2 + CO \quad (c)$$

$$RCOCl + 2(C_2H_5)_2NH \rightarrow RCON(C_2H_5)_2 + (C_2H_5)_2NH.HCl \quad (d)$$

Equations a, b and c are from Adams and Ulich (1920).

4.1 Synthesis of ^{14}C-DEDA and -DEPA.

Five grams each of decanoic and palmitic acid were refluxed with twice the theoretical quantity of thionyl chloride for two hours. The excess thionyl chloride was removed with the aid of a dry vacuum from a water suction pump (dried through a calcium chloride drying tower) and gentle heating on a water bath at 50°C. The acid chlorides thus produced were further purified by distillation.

11.13mg 1-^{14}C-diethylamine hydrochloride was dissolved in 0.4ml anhydrous pyridine. To this was added twice the theoretical quantity of the appropriate acid chloride as prepared above. The reaction mixture was allowed to stand for three weeks in a desiccator over calcium chloride. The pyridine was then removed as the hydrochloride in 5% HCl and the molluscicide taken up in diethyl ether.

The molluscicides were further purified by passing the ether solution through an aluminium oxide column to remove the excess acid. Various types of alumina were tried for this purpose and the best was found to be Technical Aluminium Oxide, Type H. This gave a yield of 95% diethylamide from a 1:1 mixture of acyl-diethylamide and the corresponding acid. Lastly the ether was removed in a stream of dry air with the aid of gentle heating on a water bath. The final product was weighed.

The purity of the amides was checked by thin layer chromatography on Merck Alumina, Type G using chloroform - methanol, 50:1 as the solvent. The spots were developed in iodine vapour and checked for radioactivity with a Phillips Type PW 4012/01 radiation detector. The molluscicides were dissolved in 10ml acetone of which 0.1ml was used for a specific activity determination. The remainder was distributed between 10 stoppered test-tubes for later use. Data and results relating to these syntheses are given in Table 22 and below.

4.1 Results.

Thin layer chromatography of the molluscicides indicated that the DEDA was pure but the DEPA was followed by a just detectable spot of the same mobility as palmitic acid. In both cases the only radioactive spots were the molluscicides.

TABLE 22

Data relating to and results obtained from the microsyntheses of ^{14}C-diethyldecanamide and ^{14}C-diethylpalmitamide.

	DEDA	DEPA
Weight of ^{14}C-diethylamine hydrochloride used.	11.13mg	11.13mg
Weight of the appropriate acid chloride used.	38.8 mg	55.9 mg
B.P. of the acid chloride.	225°C at Atm.	185°C at 8mm Hg.
Theoretical yield.	23.0mg	31.0mg
Final yield.	21.2mg	33.0mg
Weight of sample used for specific activity determination.	0.212mg	0.33mg
Specific activity.	2.40×10^6 DPM/mg	1.33×10^6 DPM/mg

4.2 Synthesis of ^{14}C-DELA.

Before using radioactive lauric acid a number of "cold" syntheses were done for practice and to determine the yield of the synthetic pathway.

Twenty milligrams of lauric acid was placed in a Quick-fit rest tube, stoppered with a calcium chloride drying tube. An excess of oxalyl chloride was added and the and the reaction mixture allowed to stand overnight at room temperature. The excess oxalyl chloride was then removed with the aid of a dry vacuum from a water suction pump and gentle heating on a water bath at 50°C.

The lauric acid chloride thus formed was dissolved in about 1ml anhydrous diethyl ether to which an excess of diethylamine was then added. The DELA thus formed was washed into a separating funnel with ether and the excess diethylamine extracted as the hydrochloride in three washings totaling 10ml 3.2%HCl. The HCl washings were themselves washed three times with ether which was added to the rest of the ether. The ether solution was dried through a column of anhydrous sodium sulphate and this column was then washed three times with fresh ether.

The ether was removed from the DELA in a stream of dry air with the aid of gentle heating on a water-bath. The molluscicide thus obtained was weighed, dissolved in acetone and the purity checked by thin layer chromatography on Merck Alumina Type G using chloroform-methanol, 50:1 as solvent. The spots were developed in iodine vapour.

Prior to the "hot" synthesis, 4.75mg of 1-^{14}C-lauric acid of an activity of 500µCi was dissolved in 50ml AR benzene containing 195mg "cold" lauric acid. This solution was divided into 10 aliquots, each containing 20mg lauric acid with an approximate activity of 50µCi.

Before commencing the synthesis the benzene was evaporated in a stream of dry air. The procedure for the hot synthesis was the same as that used in the preliminary syntheses. The ^{14}C-DELA thus produced was checked for purity by thin layer chromatography and the spots checked for radioactivity as before.

The specific activity of the molluscicides was determined in 15ml DTN. The counts were corrected for quenching and also for the ^{14}C counting effiency of the counter, all by Olivetti Programma 101. (See Appendix I for the programme used.)

4.2 Results.

Thin layer chromatography indicated that the DELA was pure and the only radioactive spot was that for DELA. Data relating to and the results of the syntheses are given below in Table 23.

TABLE 23

Data relating to and results of the microsynthesis of ^{14}C-diethyllauramide.

	Cold synthesis	Cold synthesis	Hot synthesis
Weight of lauric acid used.	20mg	20mg	20mg
Weight of oxalyl chloride used.	100mg	100mg	100mg
Weight of diethylamine used.	100mg	100mg	100mg
Theoretical yield.	25.5mg	25.5mg	25.5mg
Final yield.	26.7mg	26.3mg	25.4mg
Colour of product.	Pale yellow	Pale yellow	Pale yellow
Specific activity.	---	---	3.55×10^6 DPM/mg

SECTION 5.

DISCUSSION.

The results of the syntheses of the radioactive molluscicides speak for themselves and the little discussion is devoted to the relative advantages of the two mwthods. The yields of both methods are above 90% and are considered good in view of the small scale of the syntheses. The synthetic pathway via oxalyl chloride is simpler and more rapid than the other method. However, the use of ^{14}C-diethylamine has the advantage that a number of different acyl chains may be attached to the radioactive diethylamine thus allowing the synthesis of a number of different molluscicides from the same radioactive starting material. On the economic side, ^{14}C-lauric acid is cheaper than ^{14}C-diethylamine.

The averageΔt of the haemolymph of the control snails in section 3.1.2 is 0.250^{o}C. This figure is of the same general order as reported for other fresh water gastropods. For example 0.25^{o}C for *Lymnaea peregra*, 0.15^{o}C for *Theodoxus fluviatilis*, 0.21^{o}C for *Viviparus fasciatus* and 0.18^{o}C for *Potamopyrgus jenkensi*. (Robertson, 1966)

The lack of change in snail weight on exposure to DELA indicates that DELA does not have an effect on the water permeability of *Bulinus tropicus* membranes in contact with the surrounding water. Similarly, the fact that there is not a change in the haemolymph Δt shews that DELA does not cause a loss of dissolved substances from the haemolymph. These results clearly indicate that the toxic action of DELA is not due to an interference with the permeability of the snail membranes as was at first thought.

The exposure times in these experiments were short, but in view of the fact that the snails shewed signs of stress (as exhibited by a marked extension of the foot and a loss of orientation) after 10 minute exposure to the molluscicide, are considered adequate. The snails were moribund after about 30 minutes exposure to 5ppm DELA which indicates that the toxic action is rapid and that lethal effects can be expected in the first hour. It was felt that longer exposure times would have given false results because of weight and salt concentration changes in dead or nearly dead snails

This is probably the cause of the results in Table 6 where there is a statistically significant decrease in haemolymph Δt after three hour exposure to DELA. This decrease is caused either by a loss of solute or by a flow of water into the snails. However, the difference in Δt between exposed and control animals is a slight $0.021^{\circ}C$ which is equivalent to a change in molarity from 0.0626M to 0.0608 M for an ionic solute such as sodium chloride. It is very unlikely that this small decrease in the concentration of haemolymph solute could be the direct result of the action of DELA, especially as it is only observed after the snails have passed through stress and have been moribund for some time. It seems likely that this solute loss or water inflow is only a secondary result of the actual action of DELA. It is certainly not the cause of the stress reaction and morbidity, although it may contribute to the ultimate death of the animals.

That the molluscicides are concentrated inside the live snails to a much higher level than in the water or in dead snails, is a further indication that they do not disrupt membranes because active accumulation implies the existance of an intact membrane system. To see whether uptake was active or passive, iodoacetic acid and DNP were used to study the effects of metabolic poisons on the uptake of DELA. Iodoacetic acid is a classic sulphydryl agent and is a general inhibitor of enzymes, by virtue of its reaction with -SH groups (Hochster and Quastel, 1963) and would thus stop any system dependent on enzymes from acting. DNP is an uncoupler of oxidation and phosphorylation (Hochster and Quastel, 1963) and would thus reduce the synthesis of ATP used as an energy source. That the uptake of DELA is much reduced by the action of iodoacetic and DNP indicates that an intact enzyme system and an energy source are essential for at least part of the uptake process.

DELA is taken up by pre-poisoned snails and by snails that have been killed by boiling, usually to a concentration of 7 to 10 times that in the water. This indicates that the passive uptake shewn by the dead snails is almost certainly a physical process. The passive uptake is not a simple solution of the molluscicide in the lipids of the cell membrane as shewn by the results with snails from which the lipids and phospholipids have been extracted.

With regard to these results, it is interesting to note the findings of Weinbach and Garbus (1969) which shew that, amongst many other substances, uncouplers of oxidation and phosphorylation have the ability to bind very strongly to proteins. It is well known that the fatty acids, also uncouplers of oxidation and phosphorylation, also bind to proteins such as serum albumin. (Lehiniger, 1964) This may account for the uptake of the molluscicides by the lipid extracted snails, but would seem to not be the case for the boiled snails, most of the proteins of which will have been denatured by boiling.

The study of the uptake of DEDA, DELA and DEPA shews that the uptake of the molluscicides is proportional to the passive uptake of the molluscicides by the snails. This indicates that the uptake of the molluscicides is only a passive process and that the "active" component is the need for mechanical energy to transport the molluscicide to other parts of the body where it may be passivly adsorbed. The source of mechanical energy is the heart and in the pre-poisoned snails the heart would not be functioning, thus explaining the lower uptake of the molluscicide by dead snails. This may also explain the differences in the uptake of the molluscicide by individual live snails and by groups of live snails, as the snails that were killed more rapidly by the molluscicide would not take up as much molluscicide as those killed slowly. The translocation of the molluscicide through the various cell membranes would be accounted for by its lipid solubility.

The uptake studies on DEDA, DELA and DEPA indicate that DEDA, although taken up to the same extent as DELA, is not as toxic inside the snail. DEPA, on the other hand, is as toxic as DELA inside the snail but is not taken up to the same extent thus accounting for the difference in toxicity between these two molluscicides. While these results have only been obtained with DEDA, DELA and DEPA, these effects probably apply to the whole series of molluscicides. Thus molluscicides with an acyl chain length of less than twelve carbon atoms are less effective because their efficacy inside the snail is lower. For a chain length greater than thirteen carbon atoms, the effectivity is reduced because the uptake by the snails is reduced.

DELA causes a reduction in the oxygen uptake of *Bulinus tropicus*. However, it is unlikely that this reduction in oxygen uptake is, in itself, sufficient to be the cause of death, especially as *Bulinus* sp. are known to survive for long periods in the absence of oxygen. During this period they probably rely on anaerobic glycolysis to supply their small energy requirements. (Alberts, 1966)

The work on mitochondria shews that the acyl-diethylamide molluscicides are not true uncouplers of oxidation and phosphorylation at low concentrations. However, from Fig. 8 the amount of DELA required for complete uncoupling of oxidation and phosphorylation is 0.08μMoles in 1.9ml. This is equivalent to 80ηMoles in 1900mg fliud or 0.042ηMoles/mg. This is ten times less than the DELA concentration in snails exposed to slightly above the LC_{50} for 17 hours. (0.45ηMoles/mg, from Table 13) This suggests that DELA is acting as an uncoupler of oxidation and phosphorylation in the snails. However, the mitochondrial results are based on studies with rat-liver mitochondria *in vitro* and there may be adsorption of the molluscicides on other cell structures *in vivo*, thus reducing the amount of molluscicide available to the mitochondria.

In rat-liver mitochondria the oxygen consumption had dropped to about 20% of the control value when oxidation and phosphorylation were completely uncoupled. When *B. tropicus* were exposed to DEIA (Figs 4 to 6) the oxygen consumption dropped to about 25 - 30% of the initial rate. Thus, if rat-liver mitochondria behave in the same way as snail mitochondria with respect to DELA, the snail mitochondria would at least be partly uncoupled at this low oxygen uptake. This could contribute to the death of the snails.

Unfortunately the comparison of the effects of DEDA, DELA and DEPA on mitochondria in Table 20 does not give a precise indication of the mode of action of the molluscicides in snails although the ratios for DEDA and DELA tend to point to uncoupling of oxidation and phosphorylation as being the possible mode of action.

DELA also has an unexpected effect on swelling, and ATP-induced contraction of swollen mitochondria. DELA has the reverse action that would be expected from its similarity to the fatty acids which are classic swelling agents. (Lehninger, 1964) A source of energy in the

form of ATP is required for DELA to cause swelling. Zimmer *et al* (1969) have shewn that "ATPases" are activated when mitochondria swell. If swelling is an energetic process as a result of the activation of these "ATPases" this could account for the rapid swelling of DELA-exposed mitochondria as DELA has been shewn to stimulate "ATPase" activity.

DELA causes an increase in the activity of mitochondrial "ATPase" and, should this be a general membrane effect and not confined to the mitochondrion, it would be to the further detriment of the snails.

The evidence suggests that the acyl-diethylamide molluscicides act in a three fold manner. They reduce the rate of oxygen uptake in the snails and they stimulate the hydrolysis of ATP by "ATPases" in the snail. The molluscicides probably also uncouple oxidation from phosphorylation, although this would *seem* unimportant in view of the fact that the snails are known to survive long periods of anaerobiasis. However the conditions of exposure to the molluscicide do not exclude oxygen and this may prevent changes associated with the assumption of anaerobic respiration from taking place. A fact that tends to support uncoupling as an important factor is the fact that many other molluscicides such as the substituted phenols and the nitro-salicylanilids (Williamson and Metcalf, 1967) are also potent uncouplers of oxidation and phosphorylation.

In any event, the net result of the action of the acyl-diethylamide molluscicides seems to be to cause a reduction in the amount of ATP available for maintaining vital and essential functions. One of the first systems to be affected by a shortage of energy would be the nervous system and this could explain the rapid onset of stress in snails exposed to DELA. It is possible that the snails die as a result of the breakdown of these secondary support systems. One of these systems is osmoregulation and it is seen to be affected after three hour exposure to the molluscicide.

DELA does not have any effect on rats when given oraly, even in quite large doses. (De Villiers, 1967) The reason for this is probably that the molluscicide is broken down into lauric acid and diethylamine by hydrolysis in the gut. These substances are non-toxic and in fact the lauric acid would be assimilated and used in the same manner as a normal vegetable fat. In snails the mode of

entry of DELA is through the membranes in contact with the water (probably the gill) and thus the molluscicide would not be broken down before being taken into the blood stream. This may account for the toxicity of DELA to snails.

The mode of action of DELA uncoupling of oxidation and phosphorylation is not precisely known but if the DELA is split into the acid and diethylamine on or near the mitochondrial membrane the lauric acid could act as a proton carrier in accordance with the theory of chemiosmotic coupling of Mitchell (1968) and Van Dam and Slater (1967). The acid would have the required lipid solubility and could thus uncouple by acting as a carrier of protons to "short" the ion gradient required for chemiosmotic coupling. This could also account for the decrease in efficacy with a decrease in acyl chain length below 13 carbon atoms because the lipid solubility decreases with decreasing chain length thus reducing the lipid solubility of the "proton carrier".

DELA may also act in a purely mechanical manner. Michell's (1968) theory that the components of the electron transport chain are arranged in a vectorial manner is very acceptable in view of the fact that these components are known to be located on, or form an intergral part of the inner mitochondrial membrane. DELA causes extreme mitochondrial swelling *in vitro* when ATP is present. As ATP is also present in the cell, this swelling probably also causes swelling *in vivo*. The swelling could cause stretching of the mitochondrial membrane which would change the spatial arrangments of the components of the electron transport chain and thus interrupt the flow of electrons. At low concentrations of DELA this might just cause a reduction in electron flow and thus respiration, while at higher concentrations the stretching might be so severe as to cause uncoupling.

DELA, like the fatty acids, probably binds strongly to proteins and, if this applies to mitochondrial protein as well, DELA could uncouple according to the theory of Weinbach and Garbus (1969). In binding to the proteins the DELA could cause structural changes in "coupling factors" thus altering their function and uncoupling oxidation from phosphorylation.

It appears that the exact mode of action of DELA in uncoupling oxidation from phosphorylation will remain obscure until the mechanism of action of the other uncouplers has been completely worked out.

The chances of improveing these molluscicides are slight. The mode of action of uncouplers is obscure and so is the action of "ATPase". Any work from this angle would be a lengthy project and, although it may be possible to improve the uncoupling or "ATPase" stimulation effect of these molluscicides, any drastic changes in the structure of the molecule may cause a reduction in uptake thus nullifying the improvements.

SECTION 6

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APPENDIX I

Olivetti Programma 101 programmes.

Three progammes were specifically designed for the work on snails, rat-liver mitochondria and ^{14}C quench correction.

The first programme was for the correction of counts obtained from quenched ^{14}C samples. The method of quench correction is the channels ratio method (Herberg, 1965). The data for the quenching curve was obtained from the previous worker (Stapleberg, pers. comm.) and the slope and intercept of the line were calculated by the least squares method using the Olivetti 101. (Olivetti, 1966) The programme and the formula on which it is based is given in Fig. 12.

The second and third programmes were designed to calculate the oxygen uptake of snails and the oxygen uptake of rat-liver mitochondria. The calculations are both based on the method of Umbreit, *et al* (1948). The programme for the calculation of snail oxygen uptake is given in Fig. 13 and that for rat-liver mitochondria in Fig. 14

The presentation of the programmes is similar to that used by Olivetti (1966).

CORRECTION OF ^{14}C QUENCHING IN DTN

PROGRAMME INSTRUCTIONS. NUMBER OF CARD...F.....

REGISTER 1	M	REGISTER 2	M	REGISTER F	M
AV		B/ −		1 ◇	
S	Std C.	C/ ↕		1 ◇	
C/ ↑		S	B	W	
F/ ↓		↓			
C/ :		B −			
C/ ↕		D/ :			
:		E/ ×			
C/ :		E +			
A/ ↑		D/ ↕			
R/ S		D/ .			
R S		A/ ↑			
D ↓		R/ S			
X		R S			
A ◇		D ↓			
1 ◇		X			
S	k_1	1 ◇			
B/ ↑		A ◇			
S	k_2	C/ ×			
B ↑		A ◇			
S	k_5	S	Wt		
C ↑		:			
A W		C ×			
S	A	1 ◇			
↓		A ◇			

CONTENTS OF REGISTERS	
M	Operational
A	Operational
R	Operational
b	Storage
B	Storage
c	Storage
C	Storage
a	Operational
D	
e	Constant
E	Constant
f	Constant
F	Programme

CONDITIONS

Channel 1= 50-850

Channel 2= 80-850

Gain = 5%

CONSTANTS ON THE CARD.

141·57 (k_3)	E/↑		
−26·009 (k_4)	E↑		
49900 (^{14}C Std)	F/↑		

OPERATING PROCEDURE

	OPERATION	OPERATION
	DECIMAL INDICATOR SETTING....4....	
1	Insert card F	The machine prints out the quench corrected count and the quench corrected count at 100% effiency
2	Depress V	
3	Enter ^{14}C standard CPM (Std C)	
4	The machine prints the counter effiency in percent.	9) Enter the weight of the sample (W)
4	Enter channel 1 background (k_1)	The machine prints the concentration of lable per weight units of the sample.
5	Enter channel 2 background (k_2)	
6	Enter K_5	To repeat, press W and return to step 7.
7	Enter the count for channel 1 (A)	
8	Enter the count for channel 2 (B)	

FORMULA ON WHICH PROGRAMME IS BASED.

$$C. = \frac{\left[\frac{(A-k_1)}{(B-k_2)} \times k_3 + k_4\right] \times 100 \times k_5 \times \frac{100}{E}}{Wt.}$$

ABREVIATIONS.

A = the counts per minute in channel 1

B = the counts per minute in channel 2

E = the effiency of the standard count at channel 1 setting.

k_1 = the background count in channel 1

k_2 = the background count in channel 2

k_3 = the slope of the quenching curve.

k_4 = the intercept at the Y axis of the quenching curve.

k_5 = the ratio of weight of lable to counts given by that weight of lable, e.g. 1mg per 10000 CPM or 0.00001.

W = the weight of the sample being counted.

Std C = Counts per minute of the ^{14}C-standard.

C = The concentration of the ^{14}C-labeled substance.

Fig. 12 A programme for the correction of ^{14}C quenching in DTN.

OXYGEN UPTAKE IN THE WARBURG APPARATUS. (FOR SNAILS)

PROGRAMME INSTRUCTIONS. NUMBER OF CARD....C.....

REGISTER 1	M	REGISTER 2	M	REGISTER F	M
A Y		1 ◊			
S	ZP	A y			
C ↑		C ↓			
A W		S	TBH		
C/ *		—			
S	A	B/ ↕			
↓		S	H		
S	B	↓			
+		C —			
B/ ↕		B/ ÷			
S	Vf	D/ ×			
B ↑		D :			
S	Wt	A ◊			
A ↑		C/ -			
D ↓		C/ ↕			
E/ x		C/ +			
B +		A ◊			
B/ ↕		C/ ↕			
F/ x		A ◊			
B/ ↕		1 ◊			
F X		y			
B/ +					
E :					
D/ ↕					

CONTENTS OF REGISTERS

M Operational
A Operational
R Operational
b Operational
B Storage
c Operational
C Storage
d
D Constant
e Constant
E Constant
f Constant
F Constant

CONDITIONS

The programme is limited to use with Bulinus tropicus at 25°C.

CONSTANTS ON THE CARD.

0·847 (d)	E/↑	0·0282 (α)	F↑
10005·8 (P_o)	E↑		
0·9161 ($\frac{273}{T}$)	F/↑		

OPERATING PROCEDURE

	OPERATION	OPERATION
	DECIMAL INDICATOR SETTING....3....	
1	Insert card C	The machine prints the total oxygen uptake the
2	Depress V	the oxygen taken up over the last time period, both figures in µL.
3	Enter the zero point (ZP)	
4	Enter the flask volume (A)	
5	Enter the manometer volume (B)	
6	Enter the fluid volume (Vf)	To repeat the calculation for the same flask return to step 8. For a different flask, press W and return to step 4.
7	Enter the weight of the snail in mg. (Wt)	
8	Enter the thermobarometer height (TBH)	
9	Enter the test manometer height (H)	

FORMULA ON WHICH PROGRAMME IS BASED.

$$-x = (ZP - TBH) + (H - ZP) \times k.$$

$$k. = \frac{\left[(A+B) - \left[Vf + (Wt \times d)\right] \times \frac{273}{T}\right] + Vf + (Wt \times d) \times \alpha}{Po.}$$

(After Umbreit et al, 1948)

ABREVIATIONS.

x = µL gas taken up
ZP = the zero point in mm.
TBH = the thermobarometer height in mm.
H = the experimental manometer height in mm.
A = the volume of the flask in µL.
B = the volume of the manometer in µL.
Vf = the volume of the fluid in µL.
Wt = the weight of the snail in mg.
d = the denaity of the snails in µL/mg
T = the temperature in °K.
α = the solubility of the gas involved in water.
Po = 760mm Hg expressed in terms of the manometer fluid being used.

Fig. 13 A programme for the calculation of snail oxygen uptake in the Warburg apparatus.

OXYGEN UPTAKE IN THE WARBURG APPARATUS, (NORMAL)

PROGRAMME INSTRUCTIONS. NUMBER OF CARD....H....

REGISTER 1	M	REGISTER 2	M	REGISTER F	M
AY		D/ :			
S	ZP	/ ◇			
C↑		A ↓			
S	T	C ↓			
D ↑		S	TBH		
A W		—			
C/ *		B/ ⇕			
S	A	S	H		
↓		↓			
S	B	C —			
+		B/ +			
S	Vf	D/ X			
B ↑		A ⇕			
S —		C/ —			
D/ ⇕		C/ ⇕			
D ↓		C/ +			
F/ +		A ◇			
D/ ⇕		C/ ⇕			
D/ :		A ◇			
F/ X		/ ◇			
B ⇕		↓			
E X					
B +					
F :					

CONTENTS OF REGISTERS

M Operational
A Operational
R Operational
b Storage
B Operational
c Operational
C Storage
d Operational
D Storage
e
E Constant
f Constant
F Constant

CONDITIONS

Can only be used for MET solution and at 30°C. May be used at another temperature if α in register E is changed.

CONSTANTS ON THE CARD.

0·0260 (α)	E↑		
273 (0° in °K)	F/↑		
10005·8 (Po)	F↑		

OPERATING PROCEDURE

	OPERATION	OPERATION
	DECIMAL INDICATOR SETTING....3....	
1	Enter card H	The machine prints out the total oxygen consumed in µL and the oxygen consumed in the last time period in µL
2	Enter the zero point (ZP)	
3	Enter the temperature in °C (T)	
4	Enter the flask volume in µL (A)	
5	Enter the manometer volume in µL (B)	
6	Enter the fluid volume in µL (Vf)	
7	Enter the thermobarometer height in mm (TBH)	To repeat the calculation for the same flask, press Y and return to step 7. For a different flask, press W and return to step 4.
8	Enter the experimental manometer height in mm (H)	

FORMULA ON WHICH PROGRAMME IS BASED.

$$-x = (ZP - TBH) + (H - ZP) \times k$$

$$k = \frac{\left[(A+B) - Vf \times \frac{273}{T}\right] + Vf \times \alpha}{Po}$$

(After Umbreit et al, 1948)

ABBREVIATIONS.

x = µL gas taken up
ZP = Manometer zero point
TBH = Thermobarometer height
H = Experimental manometer height.
A = Volume of the flask
B = Volume of the manometer.
Vf = Volume of the fluid in the flask
T = The temperature in °K
α = Solubility of oxygen in the fluid
Po = 760mm Hg expressed in terms of the manometer fluid.

Fig. 14 A programme for the calculation of oxygen uptake in the Warburg apparatus.

APPENDIX II

The effect of DELA on the oxygen uptake of *Bulinus tropicus*.

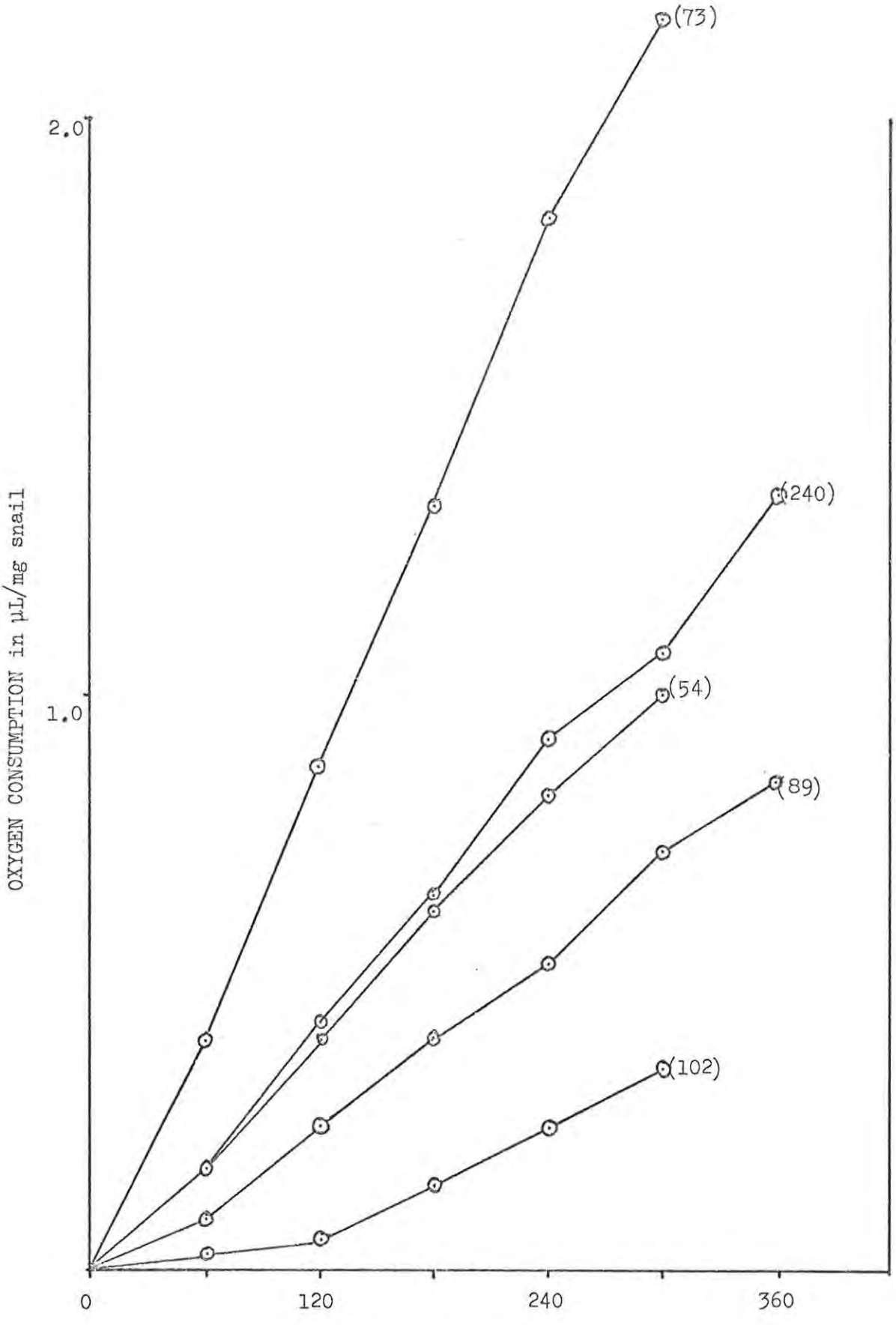
The figures presented here in this Appendix are the results of preliminary experiments done before those reported in section 3.3.2 as well as replicates of those reported there.

Prior to exposing snails to DELA a number of trial runs to measure oxygen uptake were made with individual snails in tap water. The methods followed were those in section 3.3.2 and the results are shewn in Fig. 15. The results shew that there is considerable variation between individual snails and that this variation does not seem to be related to the weight of the snails.

Figs 16 and 17 shew the effects of different concentrations of DELA on the oxygen uptake of individual snails. The rapid initial drop in the rate of oxygen uptake is thought to be due to the acetone used to prepare the DELA suspensions. This initial drop was not seen when ethanol was used to prepare the suspensions. Single snails have a low oxygen uptake and in order to increase uptake a number of snails were exposed together in the later experiments. This resulted in reduced error because the distance between successive readings on the manometer was greater.

Figs 18, 19 and 20 are the results of replicate experiments for the results shewn in Figs 4, 5 and 6 respectivly. These results confirm that after the addition of DELA there is a drop in the rate of oxygen uptake. With the exception of Fig. 16 the rate of oxygen uptake decreases to approximately 33% of the previous rate. The smaller drop in Fig. 16 is probably due to the small amount of DELA used in this flask.

Fig. 15. The uptake of oxygen by *B. tropicus* at 25°C.



The values in parentheses on the graph are the snail weights in mg.

Fig. 16 The effect of 0.05µMoles of DELA on the uptake of oxygen by *B. tropicus* exposed in 2.5ml tap water at 25°C.

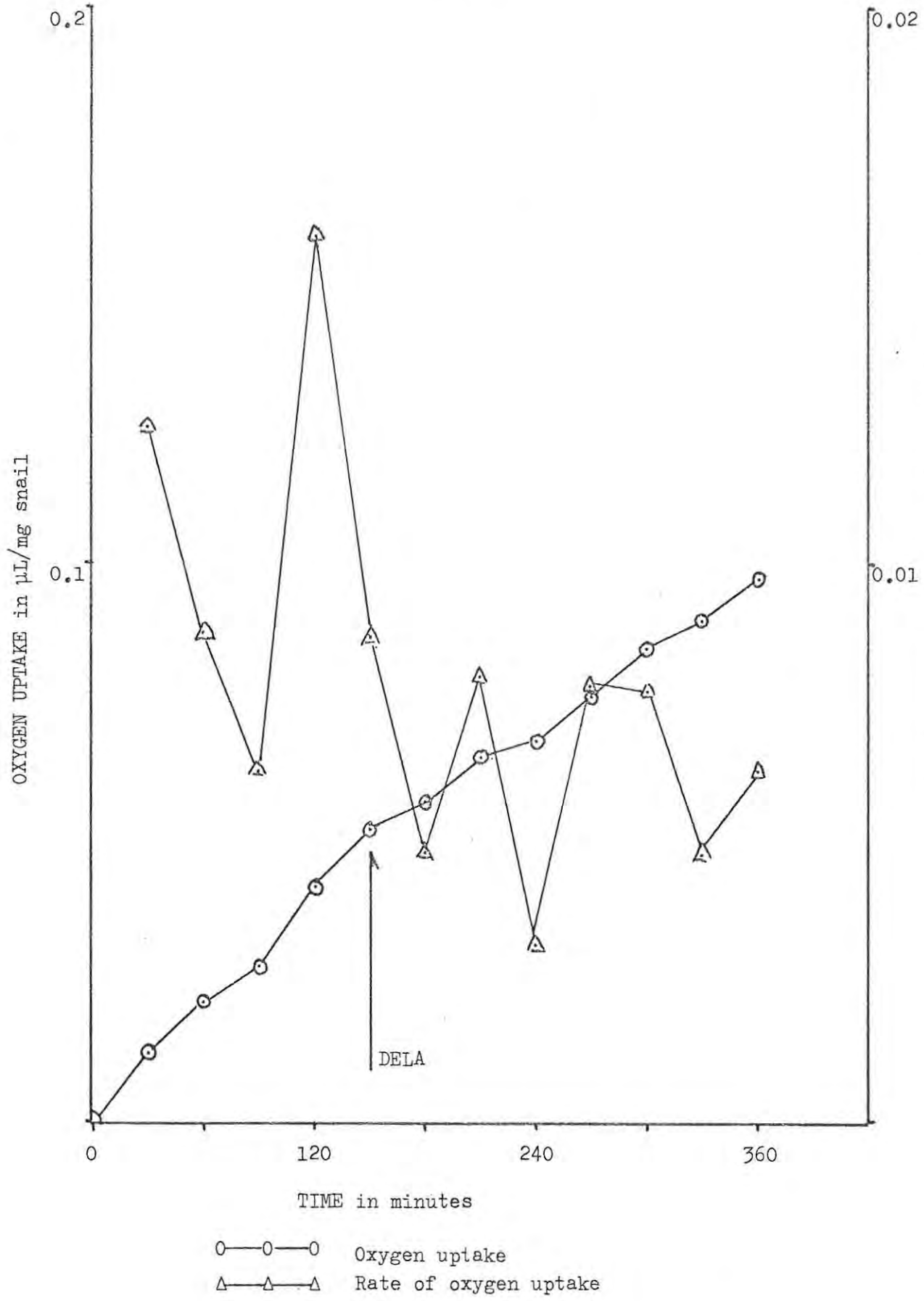


Fig. 17 The effect of 0.1μMoles of DELA on the uptake of oxygen by *B. tropicus* exposed in 2.5ml tap water at 25°C.

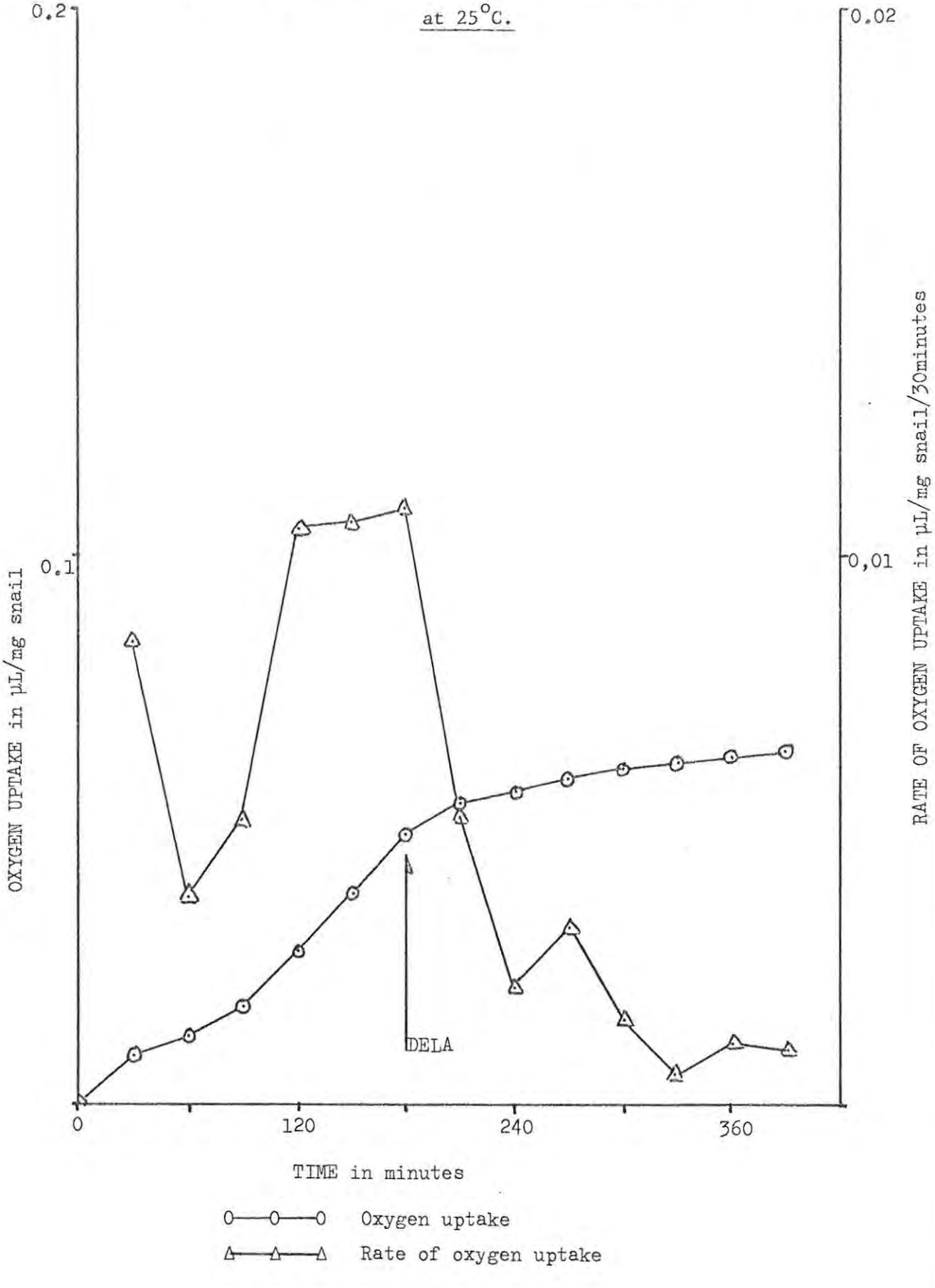


Fig. 18 The effect of 0.6µMoles of DELA on the uptake of of oxygen by four *B. tropicus* exposed in 2.5ml tap water at 25°C

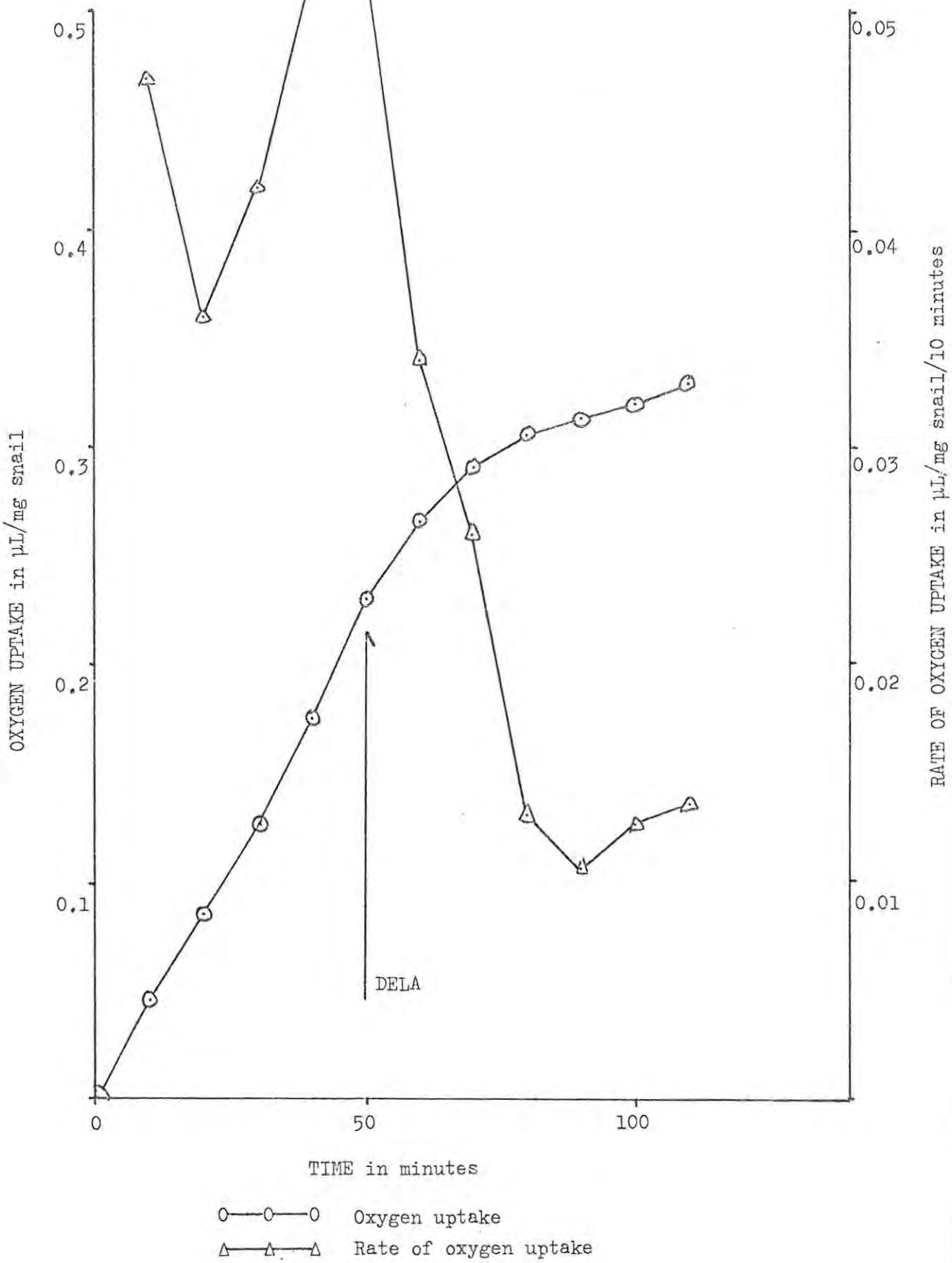


Fig. 19 The effect of 1.0µMoles of DELA on the oxygen uptake of four *B. tropicus* exposed in 2.5ml tap water at 25°C.

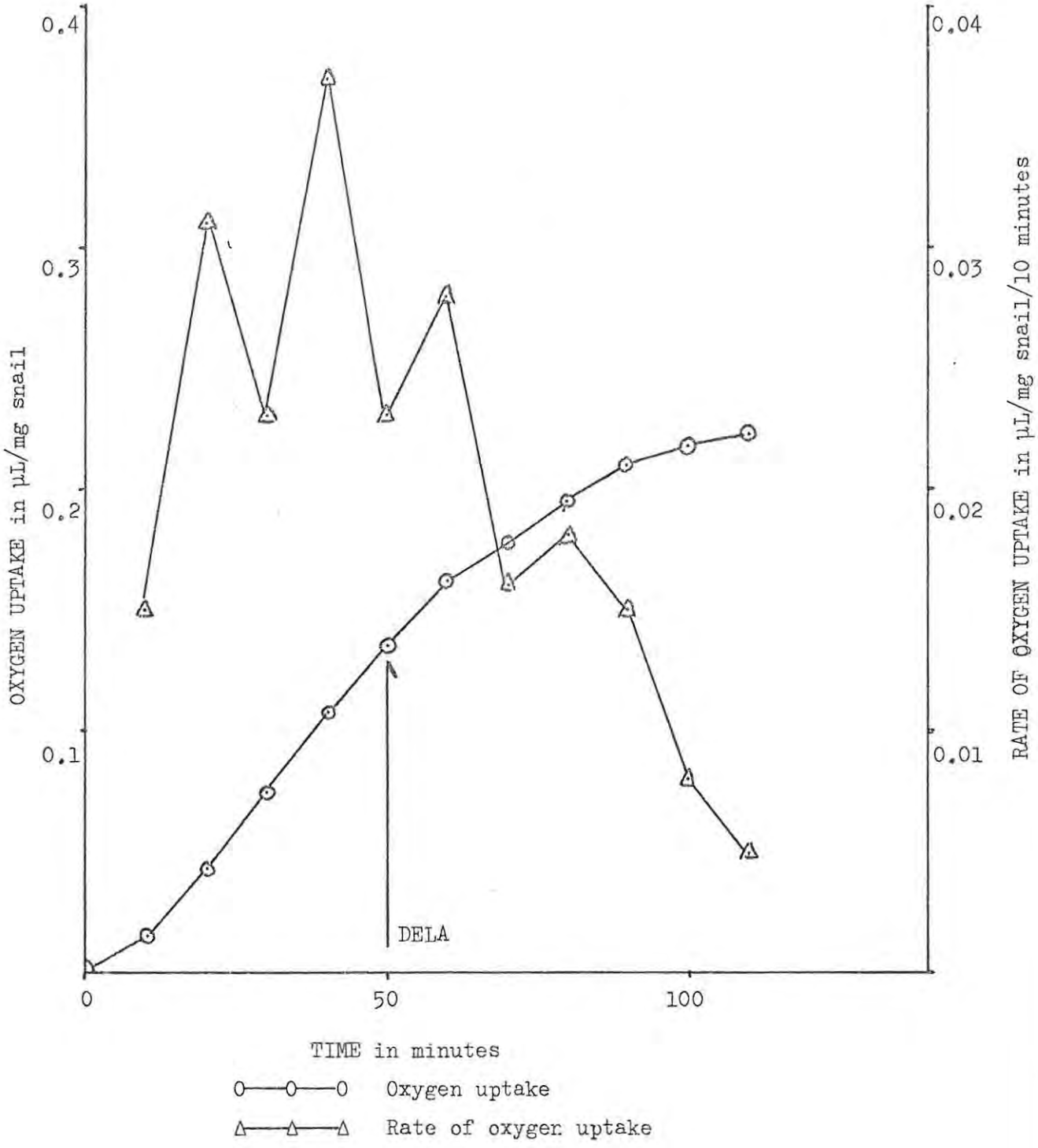


Fig. 20 The effect of 2.0uMoles of DELA on the oxygen uptake of four *B. tropicus* exposed in 2.5ml tap water at 25°C.