BIOCHEMICAL INVESTIGATION INTO THE TOXIC SALIVARY SECRETION OF THE TICK, *ORNITHODOROS SAVIGNYI* AUDOUIN (1827)

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF SCIENCE IN AGRICULTURE

IN THE

FACULTY OF AGRICULTURE
(DEPARTMENT OF BIOCHEMISTRY)
UNIVERSITY OF PRETORIA
PRETORIA

MAY 1976

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ACKNOWLEDGEMENTS

The author wishes to thank the following :

Professor D J J Potgieter for his interest, invaluable aid and guidance.

Dr C J Howell and his Department for their help in collecting many thousands of ticks and the salivary secretion.

Dr V de Villiers for his advice and help during the initial stages of the investi= gation.

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LIST OF SYMBOLS AND ABBREVIATIONS

A	area under schlieren peaks (ultracentrifugation)
APDT	Acetyl-L-phenylalanyl-L-diiodotyrosine
AR	analytical reagent
ATEE	acetyl-L-tyrosine ethyl ester
BAEE	benzoyl-L-arginine ethyl ester
c	concentration (ultracentrifugation).
CM-	carboxymethylated
DEAE-cellulose	diethyl amino-ethyl cellulose
DTNB	5:5-dithiobis-2-nitrobenzoic acid
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetic acid
ε	molar extinction coefficient
θ	phaseplate angle (ultracentrifugation)
J	interference count across a boundary (ultracentrifugation)
j	interference fringe number (ultracentrifugation)
M	molecular mass
mA	milliampere
OD	absorption
ρ	solution density
R	universal gas constant
r	radial distance in cm, corrected for camera lens magnification (ultracentrifugation)
rpm	revolutions per minute
RNase	ribonuclease
PITC	phenylisothiocyanate
PTC	phenylthiocarbamyl
PTH	phenylthiohydantoin

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T	absolute temperature
TMS	N,O-bis(trimethylsilyl)acetamide
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
V	volt
υ	partial specific volume
W	mass per cent
ω	angular velocity

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CHAPTER 1

INTRODUCTION

Ticks were suspected of being vectors of disease as early as the beginning of the last century. In 1825 it was believed that scrub ticks might have been involved in the transmission of tick paralysis to calves in Australia (1). In the Republic of South Africa the bont tick was suspected of being responsible for the transmission of heartwater as early as 1838 and in 1869 in the United States of America ticks were found to be associated with Texas fever (1).

The first confirmed instance of the transmission of a protozoan parasite by an arthropod was reported by Smith and Kilbourne in 1893 (2). They found that the cattle tick, (Boophilus annulatus) was the intermediate host of Babesia bigemina. After this significant discovery a great variety of causal disease agents became known to be transmitted by members of the families Ixodidae Murray and Argasidae Canestrini. Ticks are now known to serve as vectors for protozoa, rickettsias, anaplasms, viruses, spirochaetes and bacteria. The various ticks involved in the transmission of diseases in livestock have been enumerated by Neitz (1).

In the natural infection cycle of arthropod-borne disease agents, the sequence of events is the intake of a pathogen from an infected vertebrate host, its development in the arthropod's tissues, and the transmission to a sus=ceptible host animal (3).

In all known diseases of which ticks are the vectors,

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the causal agents have been described and trans-stadial and transovarial development have been established with the exception of tick toxicoses in which pathogenic agents are evidently not involved (2). Thus, the nature of the causal agents associated with tick paralysis, sweating sickness and tick toxicoses caused by the Sand tampan have not been established (1, 4). Tick paralysis and sweating sickness are not transferred from affected to healthy animals by means of blood or organ suspensions. Further= more, the causal agent is retained by the ticks, irrespec= tive of whether they feed on susceptible, immune or insus= ceptible animals. Ticks infected with a pathogenic agent can transmit the particular disease transovarially for many generations. However, the causal agent of tick toxicoses appears to develop only in the invertebrate host and time= ly removal of ticks from vertebrate hosts is followed by a subsidence of the symptoms. Recovered animals develop a durable immunity (1, 5). These observations regarding tick paralysis and sweating sickness are most probably also applicable to tick toxicoses caused by the Sand tampan.

Ross (6) was the first to provide experimental evidence suggesting that tick paralysis may be caused by a toxin. He showed that salivary gland extracts of Ixodes holocyclus, when injected into mice, gave rise to symptoms resembling those of tick paralysis. Furthermore, he found that the toxic effects could be prevented by a serum ob= tained from dogs on which large numbers of these ticks had been allowed to engorge. Kaire (7) described the prepara= tion of a partially purified toxin capable of causing tick paralysis, from homogenates of replete I. holocyclus.

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The purification method involved chromatography on DEAE-cellulose columns. However, no information regarding the chemical nature of the toxin was reported except, that the toxin was found to be resistant to the action of pepsin, trypsin and papain and that it was distinguish=able from the blood anticoagulant prepared from I. holocyclus. Since the toxic fraction was isolated from whole tick homogenates, no direct evidence that the toxin was trans=mitted by means of the salivary secretion was provided. Un=substantiated claims of toxigenic, lytic and blood anti=coagulant activities present in salivary secretions of ticks have been made (8). However, the first unambiguous evidence in support of a toxigenic agent was recently reported by Neitz, Howell and Potgieter (9, 10, 11). These workers succeeded in isolating chemically a pure toxin from the salivary secretion of Ornithodoros savignyi. In addition, they described various enzymatic activities present in the secretion as well as some physical and chemical properties of the secretion (12).

Hellmann and Hawkins (13) have described blood anti=coagulins and fibrinolysins from Ornithodorus moubata. Their report is important since they differentiated between anticoagulants of salivary and gut origin, thereby clearly demonstrating discrepancies between work based on whole tick homogenates and salivary secretions. Tatchell and Binnington (8) have described the isolation of pharmocologically active material from the salivary secretion of Boophilus microplus. This material caused contraction of rat fundus preparations and an increase in capillary permeability.

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The above-mentioned findings provided the first evidence that biologically active compounds are indeed present in the salivary secretions. These active components are most like= ly transmitted during the feeding of the ticks on their hosts and may play an important role in the syndromes produced in the hosts.

A comparative study of the chemical components and their biochemical activities present in the salivary secretions and other body fluids and tissues of various species of ticks which transmit infectious diseases or cause tick toxicoses, may provide a basis for research aimed at prophylactic and/ or therapeutic treatment (4). Furthermore, the free amino acid pattern of the salivary secretion could, like the free amino acid pattern of insect hemolymph, serve as a taxono= mic character (14, 15). In addition, valuable information concerning the intermediary metabolism of ticks may be obtained. For instance, Frayha _et al_. (16) made an inten= sive study of the chemical composition of the coxal fluid of the argasid tick _Ornithodoros savignyi_. The compounds studied were electrolytes, amino acids, proteins, nucleic acids, carbohydrates and lipids. The results revealed the existance of important correlations between the chemical composition of body fluids and intermediary metabolism. Hamdy (17) also stressed the importance of an extensive knowledge concerning the chemical composition of hemato= phagous arthropod excreta in evaluating the epidemiological significance of pathogens and the vector's digestive cycle and metabolic pathways.

A detailed study of the intermediary metabolism of ticks is of importance also in the investigation of the

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biochemical effects of acaricides. It is inevitable that an ultimate understanding of resistance developed by an increasing number of tick species to certain acaricides will have to be sought at a metabolic level. In addition, a knowledge of the metabolic fate of these compounds could aid in the development of alternative, and perhaps more potent acaricides. Saliba et al. (18) have worked towards this goal by investigating the effects of a phosphorothionate (ronnel) on the metabolic rate of Ornithodoros savignyi.

The protein pattern in the salivary secretion, salivary glands, hemolymph and coxal fluid has been reported for a number of tick species (8, 9, 16, 19). Van Sande & Karcher (19) reported that the protein pattern in the hemolymph of ticks infected with pathogenic organisms was similar to that of normal uninfected individuals. Furthermore, the pattern was not influenced by the species of the host on which the ticks fed. It should be of interest to know whether these findings will be applicable to the salivary secretions.

The undoubted importance of a detailed study of the chemical and biological activity of body fluids and tissues of ticks led to the decision to investigate the salivary secretion of the Sand tampan tick. The selection of this species depended in part on the economic problem it presents over a large area in the Republic of South Africa. In addition, the selection was influenced by the relative ease of collecting fairly large number of ticks (20), as well as the fact that the salivary secretion of these ticks may be procured by means of a well-established method (4).

A short survey of unique and some interesting facts about the Sand tampan tick may be useful in providing a

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broader and perhaps even relevant biological background to the above-mentioned biochemical investigation. Much of the material for this survey comes from the observa= tions of Theiler (21).

Sand tampans are responsible for considerable stock losses in the sandveld areas of the North West Cape, Kalahari and South West Africa (20). The tick has been recorded in many parts of the world, noticeably in the Central and Near East and in the drier areas of Africa. In the Republic of South Africa the ticks are prevalent in the districts of Gordonia, Kuruman, Vryburg and Mafeking; in the northern areas of Namaqualand, Kenhardt and in the southern parts of Botswana and South West Africa, throughout the Kalahari Sand-belt of Keetmanshoop, Gibeon and Gobabis up to the level of the Epukiro River. Most of these areas in the North West Cape and South West Africa have an annual rainfall of less than 40 cm per annum. The sand in the Kalahari may be between 1000 and 1500 cm in depth, overlying a continuous sheet of calcareous limestone and grits.

Ornithodoros savignyi occurs mainly in deep sand in the shade of large-crowned trees, especially the Kameeldoring Acacia giraffae, the Vaalkameel Acacia haematoxylon, the Witgat Boscia albitrunca and the Rosyntjiesbos Grewia flavia. The tampan avoids sunlight and sand exposed to the direct rays of the sun. Under field conditions the tick has been reported from approximately 8 cm downwards and can move rapid= ly through loose dry sandy soil. In moist soil the movement is restrained.

Since the tampan is a quick feeder (first stage nymphs engorge in 10-14 minutes and later stage nymphs and adults in 15-30 minutes), it is difficult to compile a host list.

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However, they are known to attack camels, cattle, mules, don= keys, sheep, goats, pigs and man. Lion, rhinoceros, buffalo and giraffe have also been reported to be attacked.

The stimulus to bring the tampan to the surface has been investigated by Nevill (20). He showed carbon dioxide to be the main stimulant which caused tampans to surface. Before these observations it was supposed that vibrations of the sand surface, caused by the mass and movements of animals was the main stimulus. These assumptions were based on observations reported by Theiler (21): "Small animals play no role, or but a minor role as hosts; their weight and movements being inadequate to cause vibrations of the earth which appear to be the main stimulus to bring the tampan above ground", and "Placing fowls under a tree in itself did not bring forth the tampan, but when the owner sat next to them, it came out quickly. This same observation has been made for small dogs".

The report of Nevill (20) is important since it advocates that tampans may be collected in large numbers for experimen= tal purposes by carbon dioxide stimulation and it also offers a means by which tampans may be controlled over confined areas.

There is little information regarding the starvation-survival periods for O. savignyi. However, it is presumed that, as with most other "desert" argasids under favourable humidity and temperature conditions, first stage nymphs may survive for six months to two years and for late stage nymphs and adults from two to eight years or longer (21). Further= more, it appears that the tampan can survive without food for several years. Thus depriving them from hosts by fencing off the shade of trees is impractical as an eradication

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method.

An intensive study regarding the pathological condi= tions resulting from Sand tampan feeding on domestic as well as laboratory animals have been made by Howell and Pienaar (22).

It should be borne in mind that the results reported in this thesis were obtained from investigations on pilocarpine stimulated secretions (4). Recently, Barker et al. (23) have shown that the electrolytic composition of tick salivary secretions obtained by infrared heat, pilocarpine injection and electrical stimulation vary considerably. These authors deduced that the highest concentrations of proteins and amino acids are found after employing the infrared heat method of stimulation. These findings are of considerable importance when the chemical composition and biological activity of tick salivary secretions are investigated. Detailed studies will necessarily have to entail investigations on secretions ob= tained by various stimulatory methods. In addition, the observations of Hajjar (24) regarding the influence of the developmental cycle of ticks on the biochemical composition of their biological fluids should be noted.

In this thesis an isolation procedure for and the charac= terization of a toxic component in the salivary secretion of the Sand tampan Ornithodoros savignyi are reported. In addition, various other biologically active components as well as an investigation into the general chemical composition of the secretion is described. A brief reference is also made regarding some chemical components and activities in the oral secretion of Amblyomma hebraeum.

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CHAPTER II

EXPERIMENTAL PROCEDURES AND RESULTS

2.1 COLLECTION OF TICKS AND THEIR ORAL SECRETION

2.1.1 Collection of Ticks

O. savignyi ticks are relatively easy to collect in large numbers by means of the dry ice method described by Nevill (20). He reported the collection of approximate= ly 4500 tampans within 120 minutes in a bowl (24 cm in diameter) which was buried to the level of the rim in the sand near a cattle kraal. Previously, tampans were collected by the laborious and time-con= suming sifting of sand samples.

Nevill (20) investigated, in a detailed study the role of carbon dioxide as stimu= lant and attractant to this tick. He re= ported that tampans are capable of detect= ing extremely low concentrations of carbon dioxide while below the sand surface. Furthermore, his findings indicated that the carbon dioxide present in the exhaled breath of higher animals is the main factor responsible for primary tampan stimulation and attraction. These findings substantiate those of Kellogg & Wright (25) who reported the role played by CO_2 in the host seeking of mosquitos, and those of Garcia (26) who showed that carbon dioxide is an attractant

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for certain ticks (Acarina:Argasidae and Ixodidae).

Recently Sauer et al. (27) made an intensive study of the chemo-attraction of the lone star tick Amblyomma americanum with respect to responses to various concentra= tions of carbon dioxide, administered by traps.

For investigational purposes, the results of which are reported in this thesis, Sand tampans were mainly collected during the warm dry summer months in the Bray area of the North West Cape Kalahari by means of traps baited with dry ice.

2.1.2 Collection of the oral secretion

Gregson (29) was the first to report the collection of the oral secretion of ticks. He showed that engorging, paralysis pro= ducing, Rocky Mountain wood ticks, Der= macentor andersoni Stiles, could be induced to secrete an oral fluid into a capillary tube placed over the hypostome. He re= ported the collection of 7 µl of fluid per tick within a few minutes after stimula= tion. His attempts to produce paralysis by injecting this secretion into lambs, dogs, mice and frogs failed, however (30). Collection of the oral secretion of Sand tampans may be readily achieved by the method described by Howell (4).

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This method is based on the approach adopted by Kato et al. (31) who physiologically stimulated the secretion of live larvae of Chironomus thummi. Howell (4) reported the stimulation of the production of the oral secretion after the introduction of a parasympathetic stimulant, pilocarpine into the haemocoele by means of a blunted needle through the genital orifice. The secretion was collected in capillary tubes placed over the hypostome and chelicirae of the ticks. He reported a linear secretion volume to mass ratio of 0,048 µl per mg of tick mass. The yield of salivary secretion in the body mass range of 123 to 215 mg per individual varied from 5,9 to 10 µl. He found no appreciable difference in the quantity of salivary secretion between engorged and unengorged females and that secretion production was affected by temperature.

The use of pilocarpine to obtain oral secretions from the cattle tick Boophilus microplus has been reported by Tatchell (32). Clarke and Hewetson (33) reported a modification of this technique. They investigated the topical application of pilocarpine and found that maximum salivary secretion production could be obtained by the application of 3 µl of 50% piloacarpine in acetone on the cuticle of the ticks. The mean volume of

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secretion collected was approximately 5,9 µl per tick for ticks with an average mass of 211 mg.

Collection of tick salivary secretion by the capillary tube method is both tedious and time-consuming and the investigation of an automatic collection method seems obvious. Binnington and Schotz (34) have described an automatic salivary secretion collection procedure, applicable to *Boophilus microplus*. They employed an automatic fraction collector for the collection of secretions of these ticks after application of 20% pilocarpine in acetone on the cuticles with a brush. The mouth parts of the ticks were passed over a cluster of fine capillary tubes through which the secretion was collected by suction. They reported that 1300 ticks could be handled in 90 minutes by one per= son employing this procedure and that an average daily collection of 12,25 ml secre= tion with an average volume of 9,5 µl per tick was possible.

A similar automatic collector has been devised by Howell (35) for the collection of the salivary secretion of *O. savignyi*. Preliminary investigations have revealed that the only suitable solvent for pilo= carpine which produces secretion via the topical application method in these ticks

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is 6N HCl (36). This seems to indicate profound differences in the composition of the cuticle of Ixodidea and Argasidae ticks.

Salivary secretions, used for investigations reported in this thesis were collected es=sentially according to the original method described by Howell (4).

2.2 SOME PROPERTIES OF THE ORAL SECRETION

2.2.1 Introduction

The freshly collected oral secretion has a clear, colourless appearance, a characteris=tic root-like odour and tends to form a persistent froth when shaken in a container. When stored at room temperature for a few days it develops a yellow colour. The secretion has an average specific gravity of 1,024, an average pH of 7,9 and a moisture content of 95,7% (12).

2.2.2 Toxicity

Cases of the death of bovines overnight as the result of attack by O. savignyi in the North West Cape Kalahari as well as laboratory observations made by Howell (4), have indicated that a potent toxin was present in the oral secretion. Results obtained from detailed studies regarding the effects of the toxin on various animals is presented below (22).

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2.2.2.1 Symptoms and pathological and histological observations

Howell and Pienaar (22) have made an intensive study of the symptoms and pathological and histological changes produced as the result of feeding of the ticks on bovine and as the result of the injection of the crude secre= tion into mice, albino-rats, sheep and gui= nea pigs. Since these authors have not pub= lished a final report only some preliminary results are reported in this section with the kind permission of the authors. The results obtained with the various animals are summarized below:

(i) Laboratory mice

The injection of 0,2 ml of salivary se= cretion into mice by the intramuscular and subcutaneous routes results in death within 6 minutes. Intravenously- treated animals die in less than 3 minu= tes.

Few symptoms are observed after the sub= cutaneous introduction of the secretion. Two types of reactions are observed. The mice appear completely normal for a few minutes after injection after which they die suddenly. Most individuals leap into the air shortly before death and die after a few convulsive movements. In the second type of reaction the animals appear

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to be slightly ruffled soon after injection. Some show accelerated respiratory movements. These soon become laboured, the animals reel about drunkenly for a few seconds and die after a few convulsive movements. Symptoms suggest respiratory failure since the heart usually remains active after all respiratory movements have ceased. Post mortal changes are usual= ly negative due to rapid death. A prominent lesion however, is a massive haemorrhage at the site of injection.

(ii) Albino rats

The subcutaneous introduction of 0,1 ml of salivary secretion into these animals results into the development of a facial irritation after a few minutes, causing them to rub the nose and face continuously with their front paws. After several hours a clear mucoid nasal discharge develops which soon becomes haemorrhagic. As the re= sult of the constant rubbing of the face and nose the face becomes covered with blood-stained mucous and evidence of impaired breathing is noted. Later they become comatose, have difficulty in maintaining an upright position and once these symptoms develop, they never

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recover. Increasing the dosage, re=
sults in a more rapid mortality.
The most prominent post mortal observa=
tions appear to be disturbance of the
vascular system reflected by congestion,
oedema and emphysema of the lungs. In
some cases haemorrhage is observed.
The subcutis, at the site of introduc=
tion of the salivary secretion invari=
ably shows light to fairly heavy loca=
lised haemorrhage and the parenchymatous
organs show congestion and localised
degenerative to necrotic changes.

(iii) Guinea pigs

The resistance of these animals to the
salivary secretion is very low and no
symptoms are produced before death.
In one instance it was observed that to
allow three female ticks to engorge on
a guinea pig caused its death (4).
Post mortal changes are confined to the
lungs and vascular system and consist
of congestion, oedema and haemorrhages.

(iv) Sheep

Clinical observations are restricted to
one animal which received a total of
7,5 ml of salivary secretion, subcuta=
neously and which died 5½ hours after
receiving the first injection.
No physiological changes were noticed.

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However, considerable local and syste=
mic changes were found _post mortem_,
which indicated increased bloodvessel
permeability.

(v) Bovine

A Fries bull of 14 months with a mass
of approximately 300 Kg was exposed to
tampan bites for a period of two hours
daily. The animal died on the fourth
day. Without exception all the bites
were confined to the area between the
coronet and fetlock on all four legs.
The most marked lesions were found in
the heart and kidney. The animal
possibly died of heart failure but the
impression is formed that the basic
lesions are related to increased vas=
cular permeability.

2.2.2.2 Determination of LD_{50}

The problem of toxicity determinations has
been stressed by Trevan (37) and Russell (38).
As editor of Toxicon, Russell has examined
some 200 papers on venoms published over
a decade and observed that at least 24 dif=
ferent techniques have been described and
used for determining lethal doses. This
obviously poses a serious problem especial=
ly as far as comparison of results of dif=
ferent investigators and the comparison of
the toxicity of different toxins are con=

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cerned.

Apart from the inherent difficulties en= countered with biological assay methods, which are usually employed for toxicity determinations, factors such as toxin activation and the effects of other com= ponents which are administered with crude toxin preparations may present additional problems. For example various enzymes present in venoms may enhance the effects produced by the actual toxin (39, 40). Many of these problems fall away once the mode of action of a toxin has been elucidated because such knowledge permits a more ac= curate determination and expression of the activity of toxins.

The quantitative determination of the toxi= city of the crude oral secretion of the Sand tampan was achieved by the method de= scribed by Weil (41) To determine the end point of the activity, serial two-fold dilutions of freshly collected secretion, in the range from 1:2 to 1:256 were pre= pared with distilled water. Three week old mice were injected subcutaneously at a dosage rate of 0,05 ml per 10 g body mass. Five mice were injected with each dilution. The highest dilution which still caused death was 1:16. A geometric delution series ranging from 1:8 to 1:64

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was selected for the determination of the LD_{50}. Ten mice were used per dilution level. The dosage rate and route were identical to those described above. Deaths were recorded over a period of 24 hours. The LD_{50} for mice for freshly collected se= cretion was found to be 0,206 ml/Kg body mass while that of three month old material, stored at 0 ^{0}C was 0,220 ml/Kg.

2.2.2.3 Thermostability of the toxic activity

A two millilitre quantity of salivary secre= tion was exposed for 15 minute periods to temperatures rising in 5 ^{0}C or 1 ^{0}C incre= ments in a waterbath (See Table 1). The temperature range was from 55 ^{0}C to 85 ^{0}C. Samples were centrifuged at 3000 g after each exposure to separate precipitates. The toxicity was determined after each tempera= ture exposure by injecting mice subcutaneously at a dosage rate of 0,05 ml per 10 g body mass, using three mice per determination. The results are shown in Table 1.

2.2.3 Some Chemical properties

2.2.3.1 Ultraviolet absorption spectrum

A Beckman DK2A ratio recording Spectrophoto= meter was employed with silica cells of 1 cm lightpath length. Because of the high ab= sorbance of the oral secretion, dilution was necessary. The absorption characteristics obtained after dilution of 50 µl of secretion

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TABLE 1 RESIDUAL TOXICITY OF THE ORAL SECRETION AFTER EXPOSURE TO THE INDICATED TEMPERATURES FOR 15 MINUTES.

Temperature (^0C)	Average survival time of mice (min.)
55	8
60	7
65	8
70	8
75	14
80	19
81	20
82	32
83	Alive after 24 hours
84	Alive after 24 hours
85	Alive after 24 hours

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to a volume of 3 ml with 0,02M Tris., 0,08M NaCl buffer at a pH of 7,9 is shown in Fig 1.

2.2.3.2 Microzone electrophoresis

Microzone electrophoresis of freshly collected oral secretion was performed at pH 8,6 (Sodium bartital buffer, ionic strength, 0,75) with a Beckman microzone electropho= resis apparatus on cellulose acetate mem= branes. A constant voltage of 250 Volt for a period of 30 minutes was found to be optimal for the separation of components detectable with Ponceau S. Stained mem= branes were scanned with a Beckman Densi= tometer. Four major bands were detected as shown in Fig 2. The bands obtained from normal bovine serum, employing the same con= ditions are superimposed in Fig 2 for com= parison.

2.2.3.3 Total nitrogen content

The total nitrogen content of the oral secre= tion was determined by the micro-Kjeldahl method described by McKenzie and Wallace (42). The results showed a content of 3,64 mg ml^{-1}. Assuming that this value represents nitrogen of protein origin, containing 16% nitrogen, the crude total protein content of the se= cretion is calculated as 22,75 mg ml^{-1}.

2.2.3.4 Non-protein nitrogen content

The non-protein nitrogen content was deter= mined by the micro-Kjeldahl method (42). on the supernatant liquid obtained after

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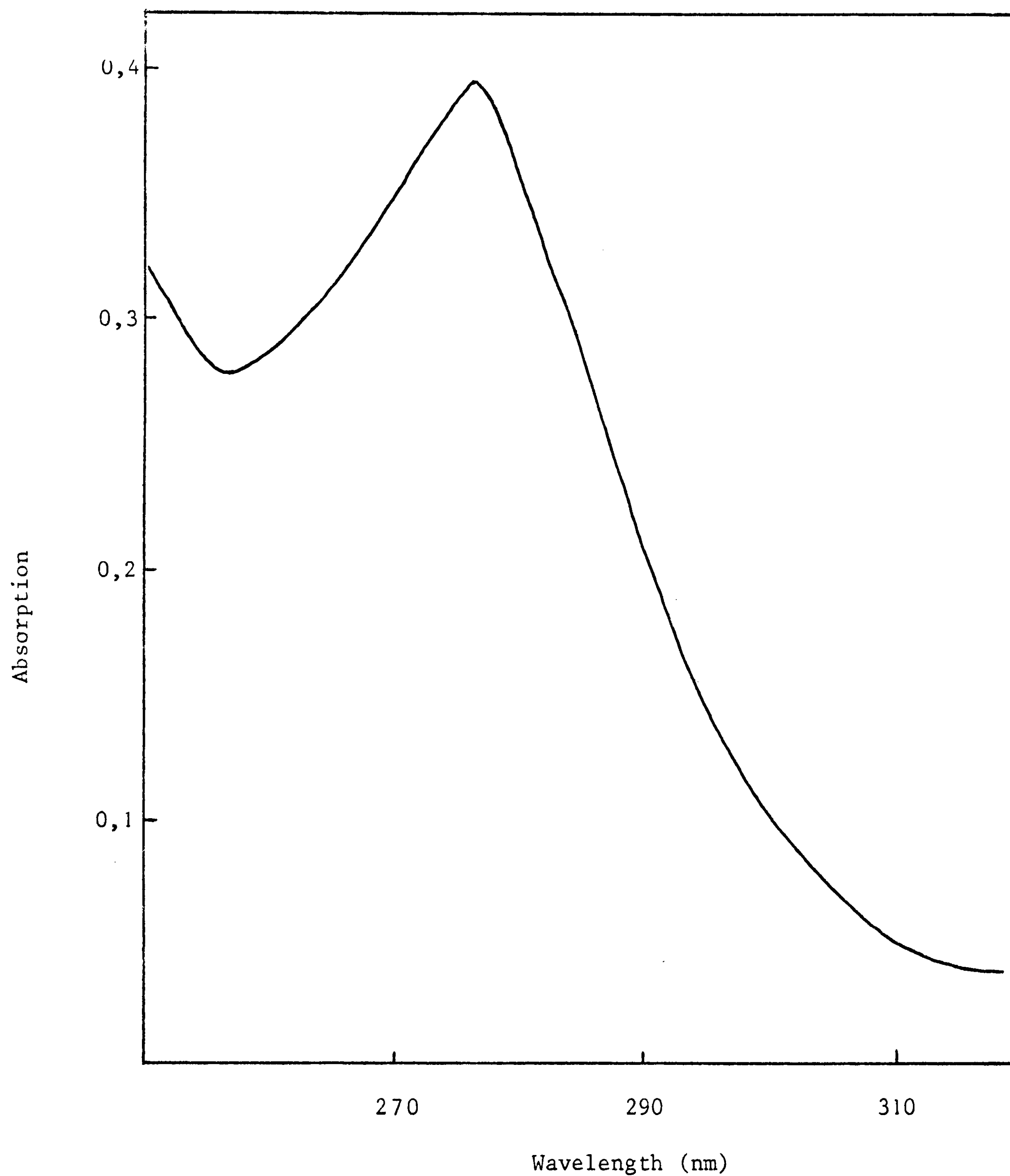

Fig 1 Ultraviolet absorption spectrum of 0,05 ml salivary secretion, diluted to 3 ml with 0,02M Tris, 0,08M NaCl buffer, pH 7,9.

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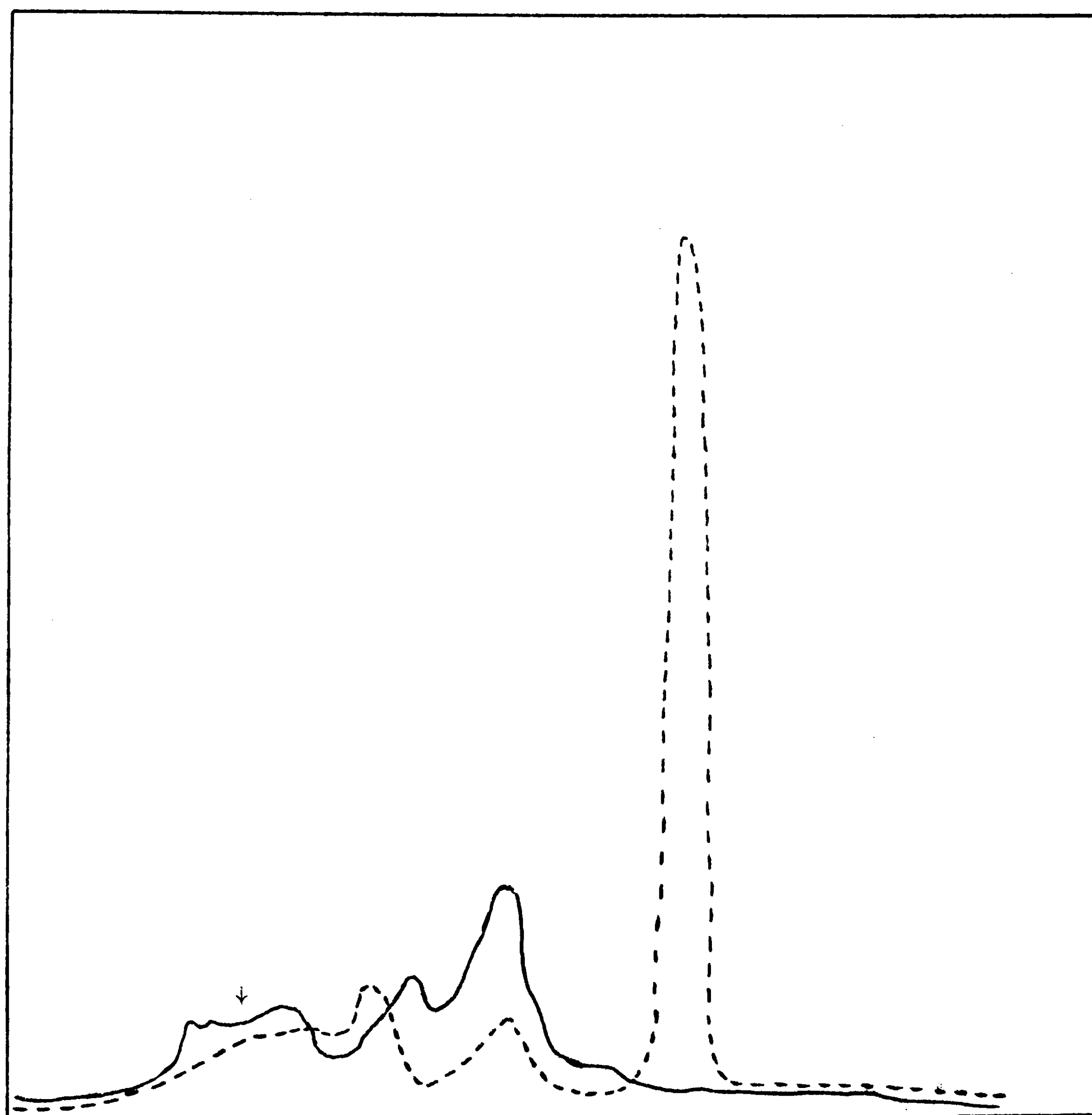

Cathode Anode

Fig 2 Electrophoretic comparison of the oral secretion with bovine blood serum. A densitomer trace of the electrophoresis strip stained with Ponceau S. The arrow indicates the origin. The dashed line represents the blood serum.

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precipitation of the proteins. The precipi= tation procedure was conducted by the follow= in method. The salivary secretion (2 ml) was diluted to a volume of 5 ml with dis= tilled water in a volumetric flask. The diluted secretion was transferred to a glass-stoppered flask and 40 ml 1% pic= ric acid added. After shaking for a few minutes, the mixture was centrifuged at 2000 g for 10 minutes and 50 ml of the clear supernatant liquid withdrawn. The picric acid was removed by a Dowex 2-X10 anionic resin in a 2x2 cm column (43). The effluent was concentrated on a rotary evaporator to 1 ml and brought to pH 7 with 1N NaOH. The solution was allowed to stand for 4 hours at room temperature to convert cysteine to cystine. After adjusting the pH to 2,2 with 1N HCl the sample was trans= ferred to a 10 ml volumetric flask and made to volume with 0,2N Sodium citrate buffer at pH 2,2. For nitrogen determination, 1,5 ml samples were used. A nitrogen value of 0,49 mg ml^{-1} was ob= tained.

2.2.3.5 Total and free amino acid content

For the quantitative determination of the total amino acids, 0,25 ml salivary secretion was in= troduced into a 15x0,75 cm glass tube and lyo= philized. The lyophilizate was suspended in 1 ml 6N HCl, after which the tube was inserted into a bath containing dry ice and acetone to freeze the sample. The tube was then evacuated with a

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water-jet pump and sealed under vacuum. Hydro= lysis was performed at 110 ^{0}C for 18, 30 and 70 hours employing 3 different samples. After the hydrolysis period, the tube was cooled to room temperature, opened and the pH adjusted to 2 with 10N NaOH. The sample was then transferred to a 10 ml volumetric flask with 0,2N sodium citrate buffer at pH 2,2 and adjusted to volume with the same buffer. One-tenth aliquots were analyzed on a Beckman Model 120B amino acid ana= lyzer (44). Tryptophan was determined spectro= photometrically on the secretion according to the method of Beaven and Holiday (45).
Methionine, determined as methionine sulfone and cysteine together with cystine as cysteic acid were determined on an oxidized hydrolyzate prepa= red in the following manner : 1 ml oral secretion sample was oxidized by the method described by Weidner and Eggum (46) and hydrolyzed with 150 ml of 6N HCl by boiling under reflux for 18 hours in an oil bath. After cooling, the hydrolyzate was filtered and concentrated on a rotary evaporator at 45 ^{0}C to a volume of 40 ml. The pH was adjus= ted to 2,2 with 30% NaOH and filtered into a 50 ml volumetric flask. The volume was adjusted to 50 ml with 0,2N sodium citrate buffer, pH 2,2. Samples of 1 ml were applied to the columns of the amino acid analyzer.
The free amino acids present in the salivary secretion were determined as described for the total amino acids on 1 ml samples of the protein-

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free supernatant liquid prepared as described in the section 2.2.3.4.

Synthetic mixtures of amino acids (Beck=man Instruments) were analyzed before and after analyses of the samples.

The values of the total and free amino acids are summarized in Tables 2 and 3, respectively. Corrections were made for the decrease of threonine, serine, iso=leucine and tyrosine (47). Proline con=tinued to increase through 70 hours of hydrolysis and this value was assumed to re=present essentially complete liberation. The increase of ammonia is adequately ac=counted for by destruction of threonine, serine, isoleucine and tyrosine. The contribution of the free amino acids to the non-protein nitrogen (0,49 mg ml^{-1}) is 0,117 mg ml^{-1}, indicating that 0,373 mg ml^{-1} of the total nitrogen in the oral secretion (3,64 mg ml^{-1}) is contributed by components other than protein, amino acids and ammonia.

2.3 ISOLATION OF THE TOXIC ACTIVITY

2.3.1 Toxicity determinations on isolated frac=tions

Samples to be tested for toxicity were dis=solved in 0,02M NaCl 0,02M Tris; pH 7,9 buffer and injected subcutaneously into Albino mice weighing ten grammes. Due to the limited availability of the oral se=cretion, it was impractical to determine the LD_{50}, as described in section 2.2.2.2,

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TABLE 2 TOTAL AMINO ACID COMPOSITION OF THE ORAL SECRETION

Amino Acids	Hydrolysis time (hours)			Corrected values	Contribution of amino acids to total nitro= gen
	18	30	70		
	mg/ml	mg/ml	mg/ml	mg/ml	mg
Lysine	2,006	1,887	2,064	1,982	0,380
Histidine	0,458	0,454	0,478	0,463	0,125
Ammonia	0,647	0,673	0,842	0,647	0,533
Arginine	0,888	0,878	0,808	0,858	0,276
Cysteic acid	1,006	-	-	1,006	0,083
Aspartic acid.	2,401	2,225	2,257	2,294	0,241
Methionine sulfone	0,435	-	-	0,435	0,034
Threonine	0,857	0,591	0,067	1,496	0,176
Serine	0,420	0,224	comple= tely des= troyed	1,078	0,144
Glutamic acid	2,777	2,512	2,671	2,653	0,253
Proline	0,087	0,103	0,105	0,105	0,013
Glycine	1,322	1,205	1,304	1,277	0,238
Alanine	0,813	0,756	0,816	0,795	0,125
Valine	1,307	1,237	1,316	1,287	0,154
Isoleucine	0,520	0,488	0,439	0,571	0,061
Leucine	1,225	1,197	1,286	1,236	0,132
Tyrosine	1,283	1,131	0,681	1,551	0,120
Phenyl= alanine	0,773	0,760	0,767	0,767	0,065
Tryptophan	-	-	-	0,692*	0,095
					3,248

* Determined spectrophotometrically.

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TABLE 3 FREE AMINO ACIDS PRESENT IN THE ORAL SECRETION

Amino acids	µg / ml	Contribution of amino acids to non-protein nitrogen µg
Lysine	98,904	18,950
Histidine	19,632	5,313
Ammonia	65,263	53,744
Threonine	42,578	5,007
Serine	4,624	0,616
Glycine	4,544	0,848
Alanine	17,641	2,773
Valine	68,269	8,165
Isoleucine	34,637	3,700
Leucine	77,933	8,323
Tyrosine	63,782	4,930
Phenylalanine	55,425	4,700
		117,074

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on all the fractions. The procedure of observing the survival time after injecting 0,1 ml of that amount of fraction present in 0,1 ml crude salivary secretion into 2 mice was employed in most toxicity determinations.

2.3.2 <u>Gel permeation chromatography</u>

Initially, a Sephadex G50 column (Pharmacia, Uppsala, Sweden) was employed for the first isolation step. In later work, Sephadex G75 and Sephadex G100 was employed. Even= tually chromatography on Sephadex G100 was found to give the most satisfactory results. With this gel, satisfactory separations could be obtained with relatively large sample volumes.

Upward-flow-elution was used in all the gel chromatographic separations reported in this thesis. The gels were packed to a height of approximately 36 cm in 40x2,5 cm columns (Pharmacia, Uppsala, Sweden).

For the initial separations a 0,08M NaCl, 0,02M Tris., pH 7,9 buffer was used as eluant. In later work the buffer was re= placed with distilled water. No apparent differences in the elution patterns or toxicity of the peaks were observed with the latter eluant when compared with buffer as eluant. Distilled water was chosen since its use obviated the need for dialysis during sample preparation for subsequent

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tion procedures and toxicity determinations. Considerable loss of toxicity during dia= lysis has been reported (9).

The gel chromatographic separations were either performed in a Beckman Model 130 Spectrochrom, monitoring at 280 and 260nm, or in a system consisting of a column, peristaltic pump (LKB Produkte) and Beck= man fraction collector. In the latter system, fractions were monitored manually in a DK2A Beckman Spectrophotometer at 280nm In all cases, the eluant flow speed was approximately 20ml h^{-1}, the column tempera= ture 8 to 10 ^{0}C and 2,6 to 2,9 ml fractions were collected.

Separation diagrams obtained after chromato= graphy of the salivary secretion on Sephadex G50, G75 and G 100 are shown in Fig 3. The yields of the separated fractions are summarized in Table 4.

2.3.3 Ion exchange chromatography

The lyophilyzed toxic peaks obtained from the Sephadex G75 and G100 columns (see Table 4) were submitted to DEAE-cellulose chromatography. Samples containing salts from the Sephadex columns were dissolved in distilled water and dialyzed against 4 changes of 500 ml of 0,04M Tris buffer at pH 7,9 over a period of 16 hours. Salt-free samples were dissolved in 0,04M Tris

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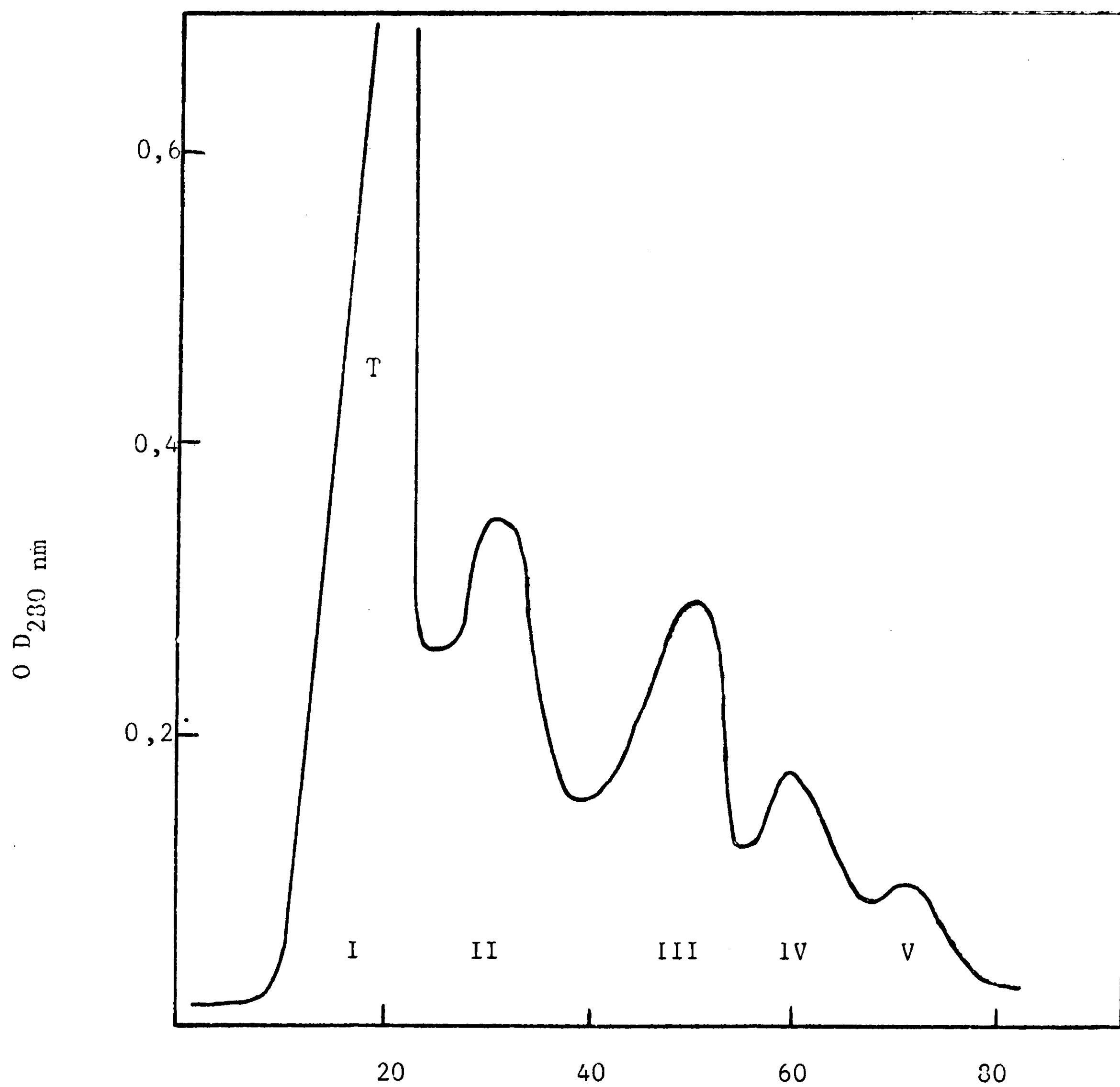

Fig 3(a) Gel chromatography of the oral secretion on Sephadex G50. Eluant : 0,08M NaCl, 0,02M Tris, pH 7,9 buffer. T indicates toxic fraction.

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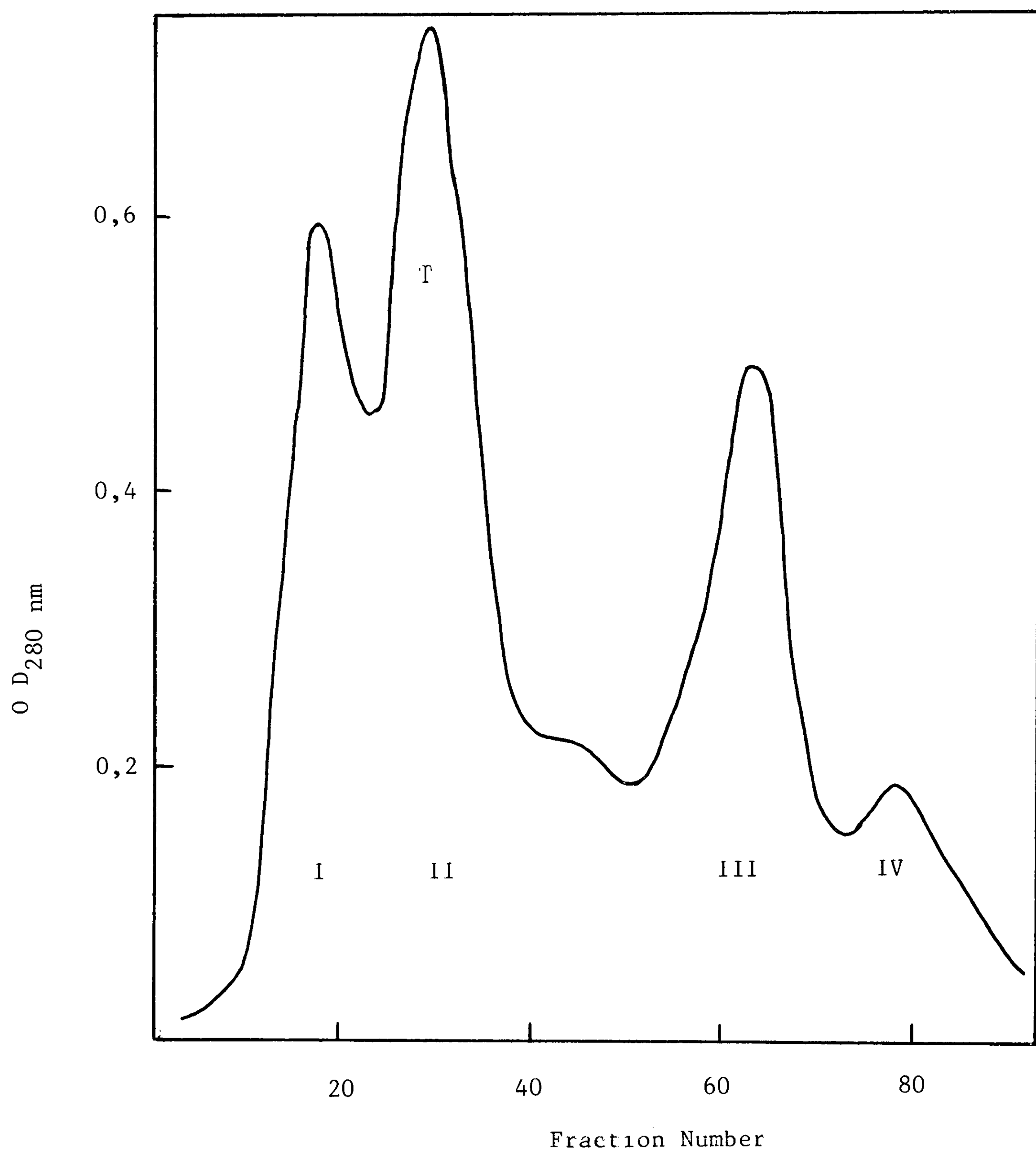

Fig 3(b) Gel chromatography of the oral secretion on Sephadex G75. Eluant: 0,03M NaCl 0,02M Tris, pH 7,9 buffer. T indicates toxic fraction.

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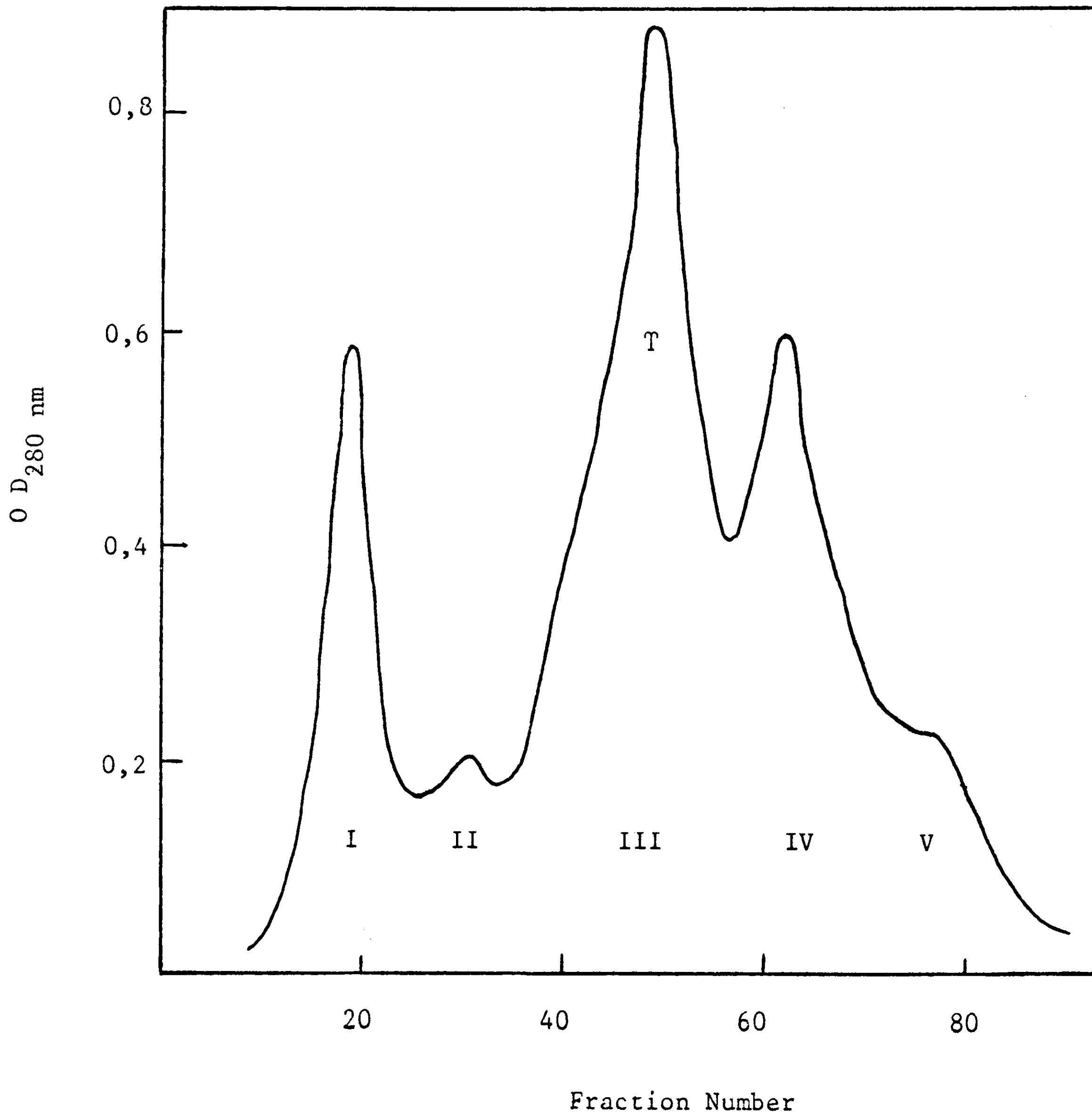

Fig 3(c) Gel chromatography of the oral secretion on Sephadex G100. Eluant : 0,08M NaCl, 0,02M Tris, pH 7,9 buffer. T indicates toxic fraction.

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TABLE 4 YIELDS OF MATERIAL UNDER PEAKS (AS % OF TOTAL) OBTAINED AFTER CHROMATOGRAPHY OF THE SALIVARY SECRETION ON VARIOUS SEPHA= DEX COLUMNS

Sephadex column	Volume salivary secretion (ml)	Peak Number (Fig 3)					Total (mg)
		I	II	III	IV	V	
G 50 *	3	80+	2	12	4	3	98
G 75 *	4	20	66+	13	1		144
G 100 *	3	3	8	50+	33	4	97
G 100 **	3	3	11	39+	46	2	97

* 0,08M NaCl; 0,02M Tris; pH 7,9 buffer used as eluant; yields calculated from spectrophoto= metric data (48).

** Distilled water used as eluant; yields calculated from gravimetric data

\+ Toxic peaks.

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buffer at pH 7,9.

The DEAE-cellulose (Cellex-D, Bio-Rad), with exchange capacity of 0,75 meq g^{-1} was prepared in the following manner : the resin (9g) was suspended in 500 ml of 0,25N NaOH and stirred for 15 minutes by hand with a glass rod. The suspension was left to stand for 20 minutes after which the supernatant, containing 50 ml fine resin material, was poured off. The washing was repeated and the suspension filtered through a sintered glass funnel with suction. After washing with dis= tilled water untill free of alkali, the resin was suspended in 0,25N HCl and treated as described for the NaOH treatment. To the resin was then added 200 ml of 0,04M Tris , pH 7,9 buffer. The suspension was stirred by hand with a glass rod for 15 minutes and subsequently poured into a column (2,5x30 cm) onto which an exten= tion tube (2,5x50 cm) had been fitted. The extention tube allowed the total resin suspension to be poured into the column at one time. The height of the packed resin was 22 cm.

Elution and monitoring of the effluent from the column was performed either with a Beckman Model 130 Spectrochrom or with a system consisting of a gradient former and fraction collector set up in the cold

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room (5 ^{0}C). The gradient former con= sisted of 2 identical perspex chambers each of 410 ml capacity, connected by means of a glass siphon. A linear gradient was established by placing 400 ml of starting buffer in the mixing cham= ber and 400 ml of 0,04M Tris., 0,16M NaCl, pH 7,9 buffer (the limiting buffer) into the adjacant reservoir. When employing the Spectrochrom a linear gradient was established over a volume of 1 litre by a suitable choice of gears on the program cam cup drive of the gradient pump.
The elution diagrams obtained after chroma= tography of the toxic fractions from the Sephadex G75 and Sephadex G100 columns are presented in Fig. 4.and the yields of the various peaks are summarized in Table 5.
Rechromatography of the toxic peaks ob= tained form the Cellex-D columns on Sepha= dex G75 or Sephadex G100 using buffer as eluant and conditions as described in section 2.3.2, showed a single toxic peak, followed by buffer salts (See Fig 5)
The progress of the purification of the toxic activity up to this stage viz after consecutive column chromatographic resolu= tion on Sephadex G75 or G100 and DEAE- cellulose is summarized in Table 6. In order to conserve material it was decided

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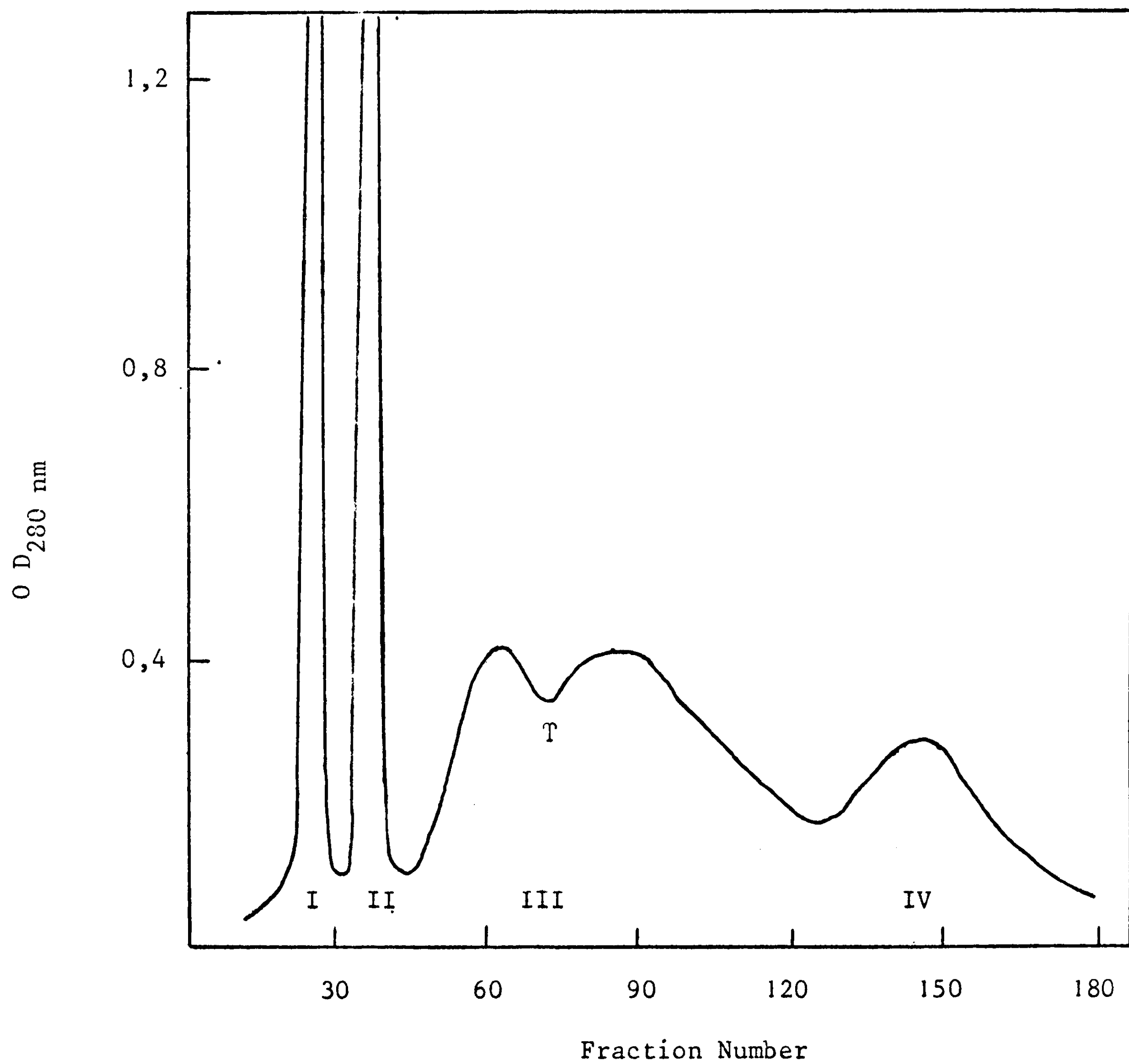

Fig 4(a) Salt gradient chromatography on DEAE-cellulose (exchange capacity : 0,75 meq / g) of toxic peaks obtained from the Sephadex G75 column (Fig 3 (b)). T indicates toxic fractions.

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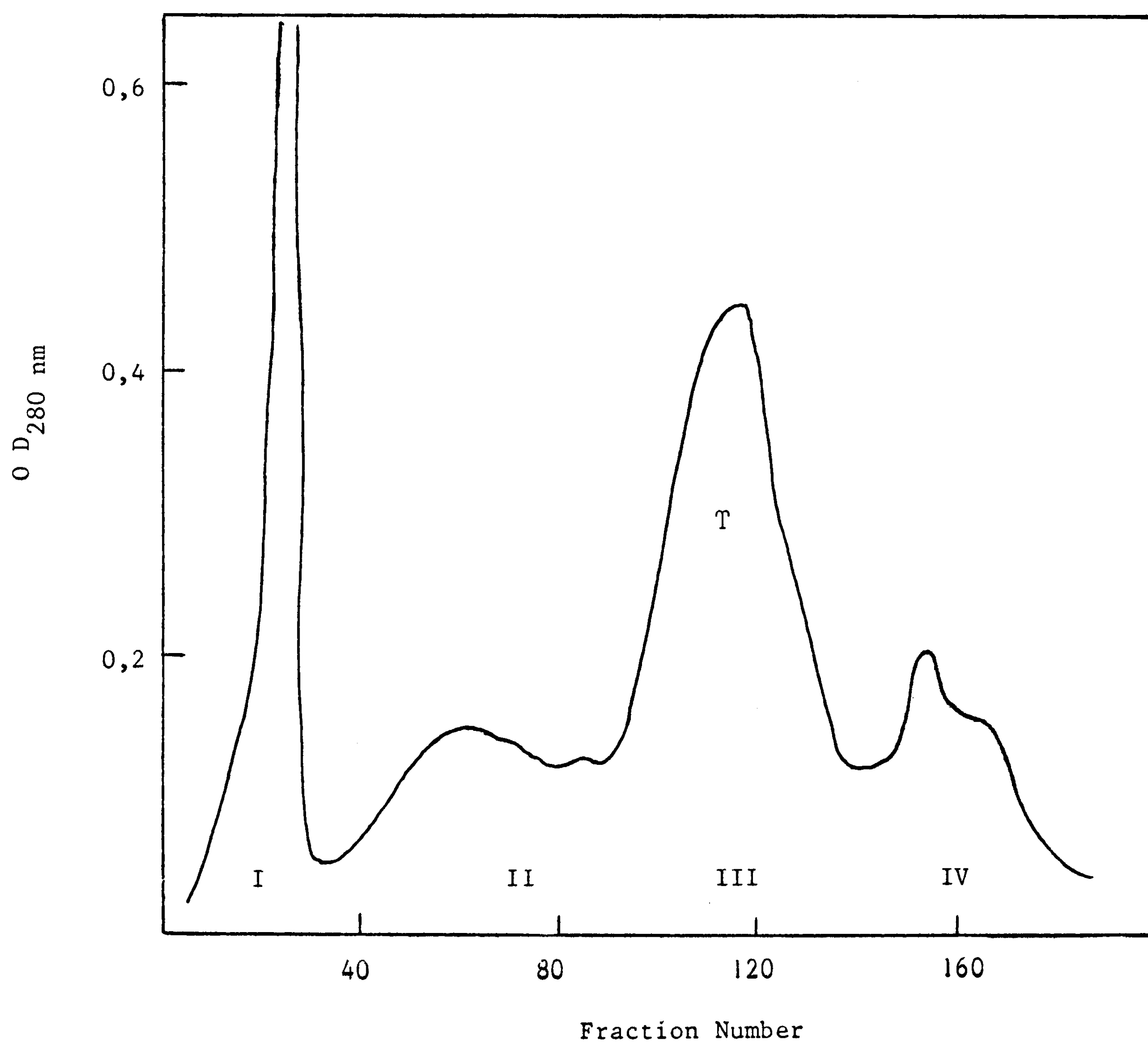

Fig 4(b) Salt gradient chromatography on DEAE-cellulose (exchange capacity : 0,75 meq / g) of toxic peak obtained from the Sephadex G100 column (Fig 3 (c)). T indicates toxic fractions.

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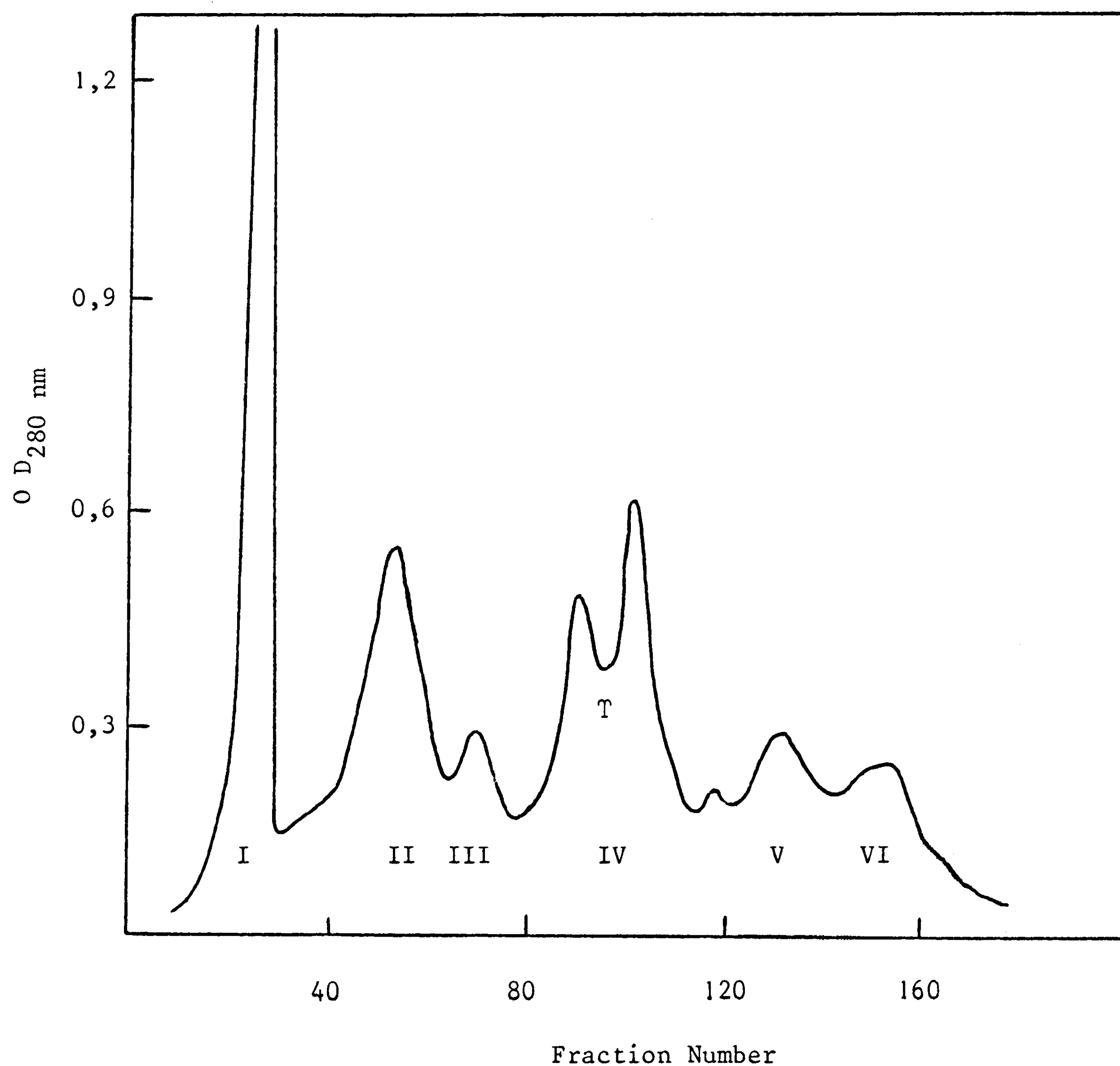

Fig 4(c) Salt gradient chromatography on DEAE-cellulose (exchange capacity : 0,96 meq / g) of toxic peak obtained from the Sephadex G100 column (Fig 3(c)). T indicates toxic fractions.

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TABLE 5 YIELDS OF MATERIAL UNDER PEAKS (AS % OF TOTAL)* OBTAINED AFTER CHROMATOGRAPHY OF TOXIC PEAKS (FROM GEL CHROMATOGRAPHY SEPARATIONS) ON DEAE-CELLULOSE

Sample	Exchange capa= city of DEAE-cellulose co= lumns (meq/g)	Peak number					
		I	II	III	IV	V	VI
Toxic peak from Sep= hadex G 75 column **	0,75	16	20	40+	22	-	-
Toxic peak from Sep= hadex G 100 column**	0,75	18	15	50+	18	-	-
Toxic peak from Sep= hadex G 100 column *f*	0,96	11	9	10	53+	10	9

* Yields calculated from spectrophotometric data (48)

** 0,08M NaCl, 0,02M Tris, pH 7,9 as eluant.

f water as eluant.

\+ toxic peaks

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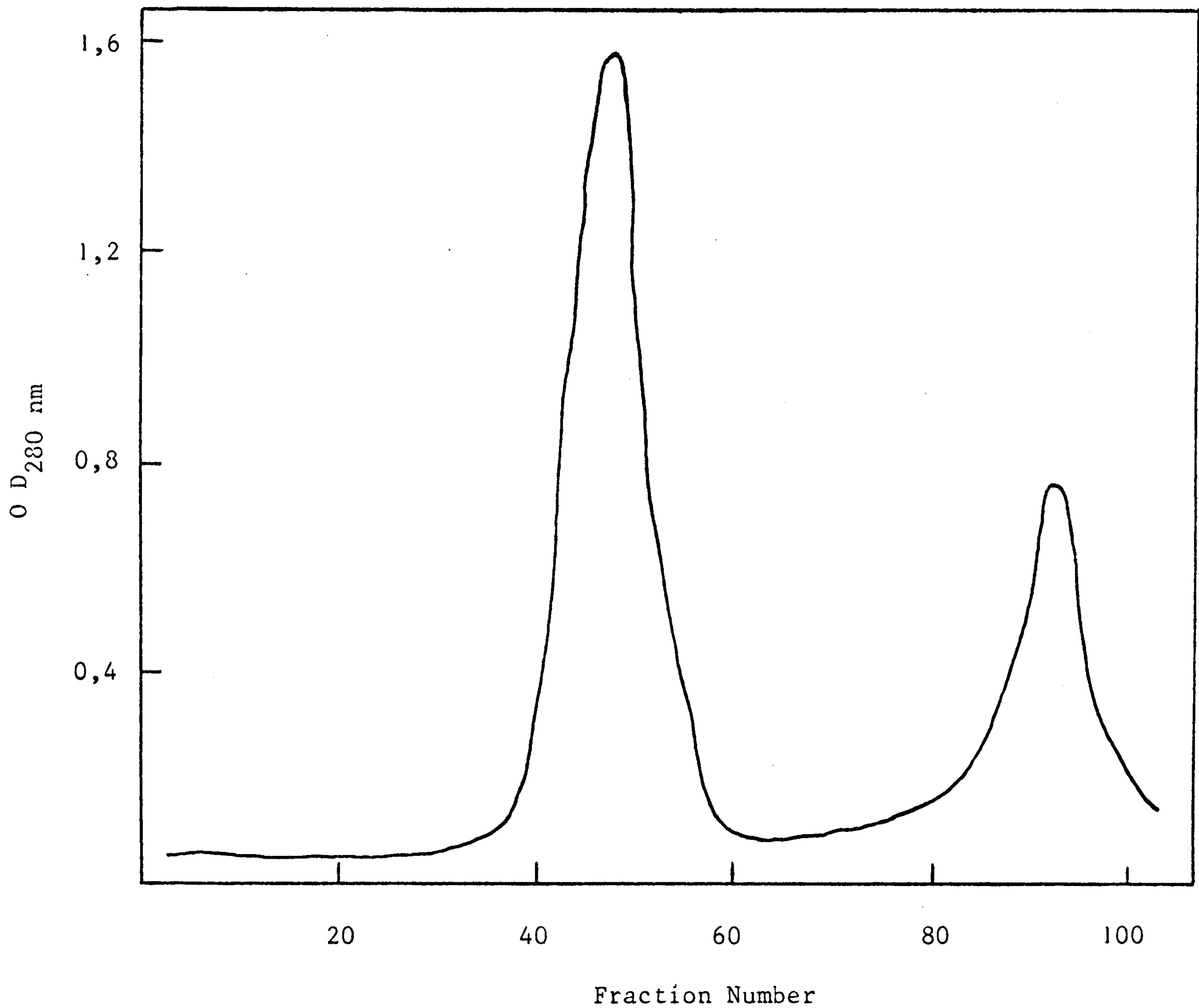

Fig 5 Rechromatography of the toxic peak, obtained from Cellex-D chromatography, on Sephadex G100 and employing 0,02M Tris, 0,08M NaCl, pH 7,9 buffer as eluant.

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TABLE 6 PROGRESS OF THE PURIFICATION OF A TOXIC COMPONENT OF THE ORAL SECRETION

Step	Total protein (mg)	Total Kg LD_{50}	$\frac{\text{Kg } LD_{50}}{\text{mg protein}}$	Enrich= ment	% Yield
Crude oral secre= tion	116	22,5	0,19		100
Sephadex G75 Chro= matography	77	18,0	0,23	1,21	80
DEAE-cellulose chromatography and rechromatography on Sephadex G75	11	9,5	0,86	4,46	42

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not to determine enrichment during sub=
sequent separation procedures.
Since chromatography on Sephadex G100
with distilled water as eluant and sub=
sequent separation on Cellex-D (ion
exchange capacity 0,96 meq g^{-1}) followed
by rechromatography on Sephadex G100
gave the most favourable separations and
yields of the toxic material, these steps
were employed in all further work.

2.3.4 Isoelectric focusing

The elution diagram of the Cellex-D column
indicated that the toxic fraction was hete=
rogeneous. At this stage of the investi=
gation, isoelectric focusing facilities
(LKB Instruments, Sweden) were available
in this laboratory. Since the heterogeneity
was most likely due to charge differences,
this technique was selected and applied in
an additional purification step (49, 50).
To determine the degree of heterogeneity
of the toxic fraction and to aid in the
selection of a suitable carrier ampholyte
range, isoelectric focusing in polyacryla=
mide gel was performed. A method, similar
to that described by Wrigley (51) was follow=

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ed. A Pleuger disc gel electrophoresis apparatus with a Duostat power supply (Beckman Instruments) was used.

The gel was prepared from the following stock solutions which were prepared as follows:

(a) 7,5 g acrylamide and 0,2 g N,N'-methylene-bis-acrylamide were mixed with distilled water and made to 25 ml with distilled water.

(b) 0,4 g β-dimethyl aminopropionitrile in 25 ml of distilled water

(c) 30 mg potassium ferricyanide in 100 ml of distilled water

(d) 480 mg ammonium persulphate in 100 ml of distilled water.

All reagents were obtained from Pleuger.

For each tube 0,48 ml of each of the stock solutions were mixed with 0,05 ml 40% carrier ampholytes (LKB Instruments, Sweden) and made to 2 ml with distilled water.

The sample, 0,04 ml containing 0,1 to 0,3 mg protein was then mixed with 1,6 ml of the gel solution and poured into the tubes. Gelation occurred within approximately one hour. For initial experiments, carrier ampholytes with a pH range of 3 to 10 were chosen and ovalbumin (Mann Research) was selected as reference and included in one of the tubes.

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The electrode solutions were 0,2% sulphuric acid at the anode side (top) and 0,2% sodium hydroxide at the cathode end. The electro= phoresis was conducted at room temperature. A maximum current of 2 mA per tube was used at a constant voltage. In a typical experi= ment with 5 tubes, the starting voltage was 25V and the current 10 mA. Within 1 hour the current decreased to 2,5 mA. The current was then increased to 10 mA by adjusting the vol= tage to 110 Volt. During the following 90 minutes the voltage was gradually increased to 250V. The current after this period was 4,2 mA and remained stable for a further time period of 1 hour after which the run was ended. The gels were removed from the tubes by gent= ly introducing water between the gel and the tube wall with a syringe and hypodermic needle. The gels were placed in test tubes containing 5% trichloroacetic acid. Protein zones were easily detected as white precipitation bands. The results with the carrier ampholytes in the pH 3 to 10 range are shown in Fig 6. Comparison of the position of these bands of the toxic sample with that of the ovalbumin standard, indicated that these bands had an isoelectric point in the region of pH 5. An experiment with carrier ampholytes in the pH region of be= tween 3 and 6 was subsequently selected to as= certain the correct choice of carrier ampholy= tes for preparative isoelectric focusing in a sucrose gradient.

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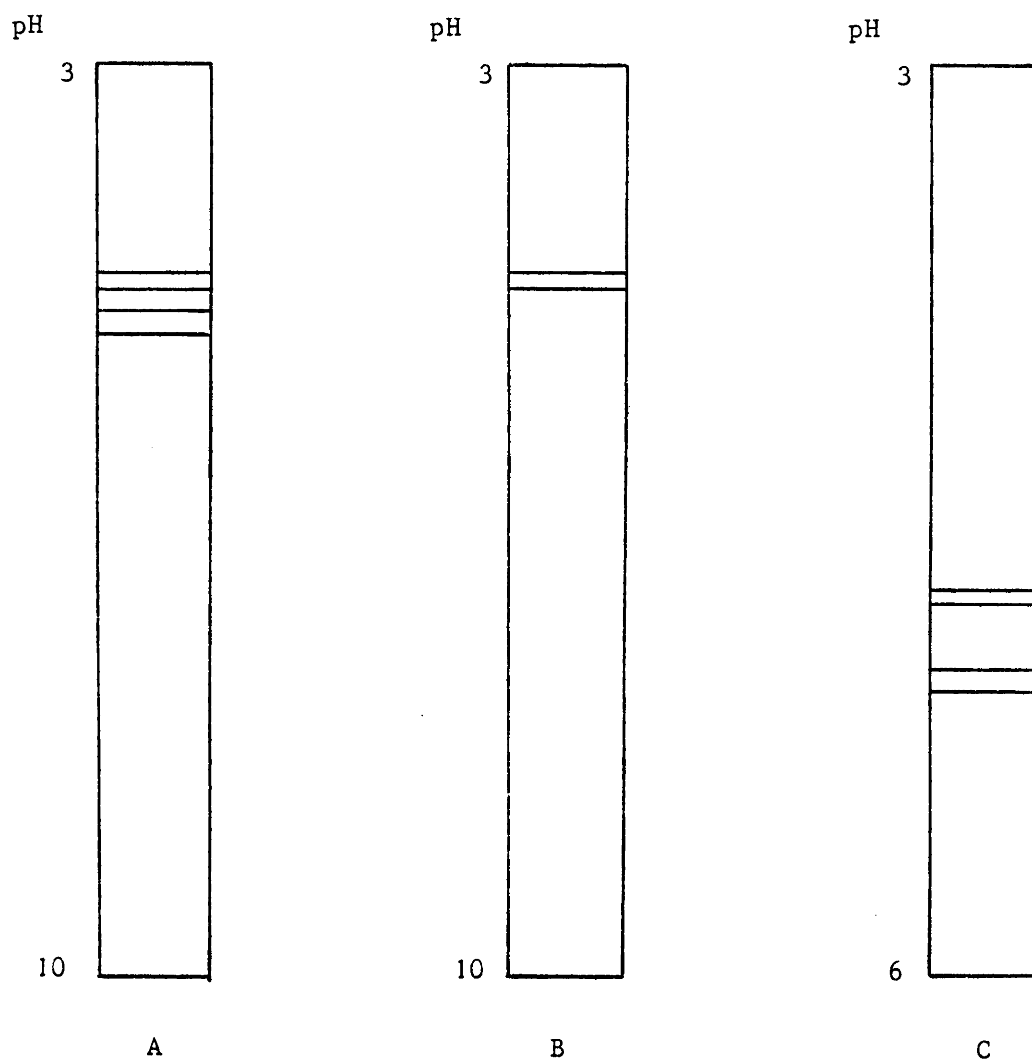

Fig 6 Polyacrylamide gel isoelectric focusing of ovalbumin (B) and of the toxic peak obtained from DEAE-cellulose chromatography (A&C)

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These results indicated that a pH gradient between 3 and 6 would provide a suitable range for the preparative separation of components of the toxic sample.
The preparative isoelectric focusing was performed in the following way. A LKB 8100 column (LKB Instruments, Sweden), of 440 ml capacity, was set up as de= scribed in the LKB 8100 Instruction Manual. The column was cooled by means of a circulating waterbath at 8 ^{0}C. The sucrose and pH gradients were formed with the LKB gradient mixer (supplied with the column) and introduced into the column with a LKB peristaltic pump at a flow rate of 3 ml min^{-1}. The final carrier ampholyte concentration in the column was 2%. The anode electrolyte solution was placed at the bottom of the column. A 3 to 6 ml sample, containing 25 to 60 mg of protein in 0,02M NaCl, 0,02M Tris, pH 7,9 buffer which contained 25% sucrose and 1% carrier ampholytes was introduced into the column after approximately 50% of the gradient had been delivered. The rest of the gradient was then layered over the sample, followed by the cathode electrolyte solution. This method of sample application was employed to prevent the sample of coming

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into contact with the electrode solutions. The power supply was subsequently connec= ted to the electrode nipples and the voltage set to 300 Volt. The initial current was 16 mA which decreased continuously during the following 27 hours. During this time period the voltage was increased manually to 600 Volt. The power was never permitted to exceed 6 Watts during these adjustments.

A final current of 7-8 mA was attained which remained constant for an additional 12 hours. After this period the power was turned off and the central electrode valve in the column closed. The electrode solution above the valve was sucked off and the column emptied with a peristaltic pump at a flow rate of 2 ml min^{-1}. Frac= tions of 2,5 ml were collected with a fraction collector. The optical density and pH of each tube was determined by means of a DK2A Spectrophotometer and Radiometer expanded scale pH meter (glass electrode), respectively. A typical plot of optical density and pH against the fraction collector tube numbers is shown in Fig 7.

Fractions electrofocused at pH 4, pH 5,1, pH 6,2, pH 8,2 and between pH 4,9 and pH 5,0 were collected and each dialyzed

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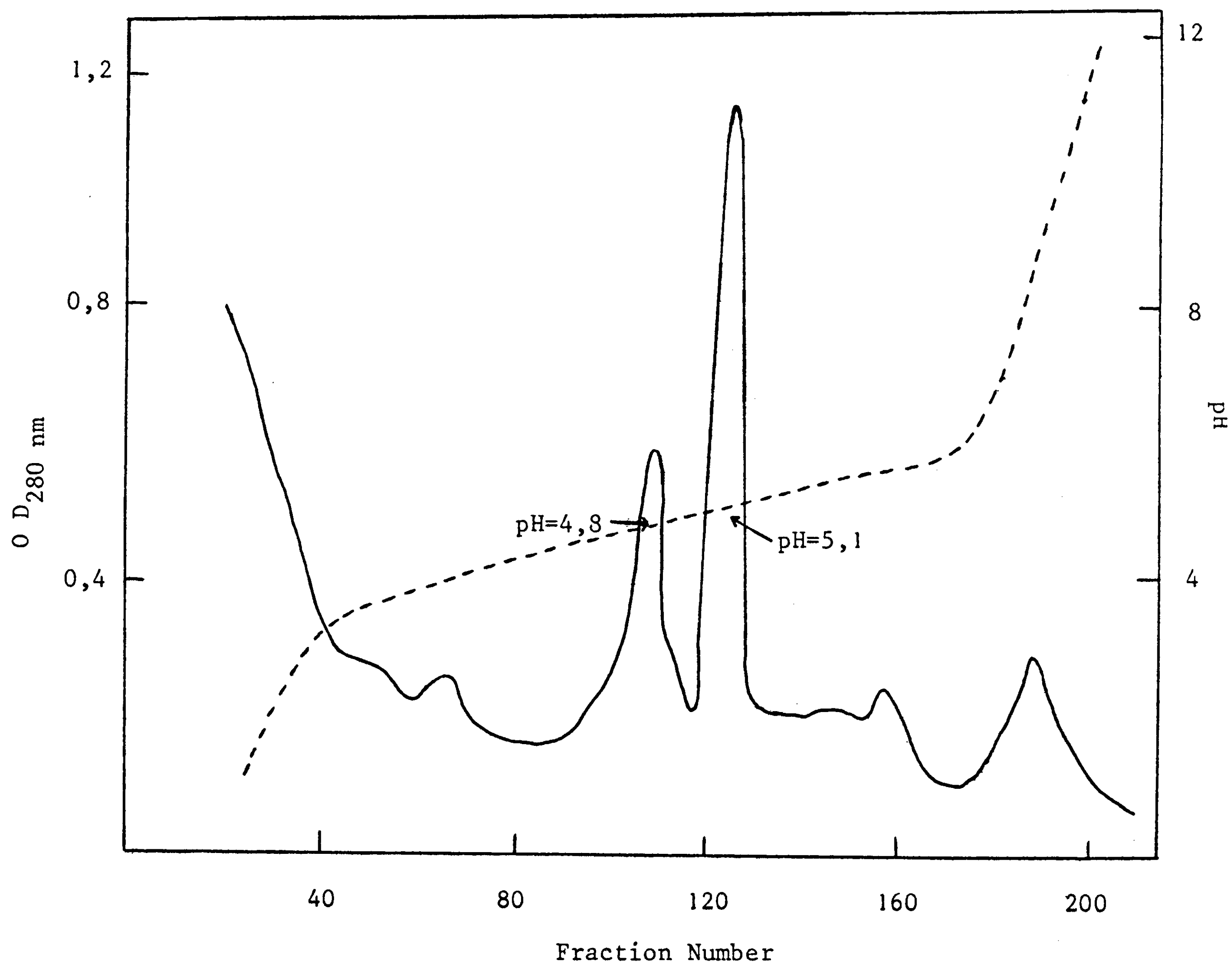

Fig 7 Isoelectric focusing of the toxic fraction obtained after DEAE-cellulose chromatography; —— indicates absorption at 280 nm and --- pH measurements.

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in cellulose dialysis casings against 1 l of distilled water at 5 °C for 24 hours. The distilled water was changed 3 times during this period. A Sephadex G100 column was used to remove the remaining contaminants after lyophilization of the dialyzed samples. The elution diagram obtained after the frac= tion electrofocused at pH 5,1 was chromato= graphed on this column is shown in Fig 8. The only toxic fraction obtained from the iso= electric focusing column, was associated with the peak with an isoelectric point of 5,1. The LD_{50} of the peak was not determined. How= ever, the subcutaneous injection of 0,4 mg in= to albino mice weighing 10 g resulted in death after approximately 90 minutes. The yield was 3,1 mg of toxic material from 1 ml of crude salivary secretion.

In later isolation procedures, the DEAE-cellu= lose chromatography step was omitted and the toxic fraction, obtained from the first Sepha= dex G100 column, directly submitted to isoelec= tric focusing using carrier ampholytes forming a pH gradient from 4 to 6. Up to 80 mg of sample were successfully separated employing this procedure. As expected, additional peaks were found but they were well separated from the toxic peak, which was electrofocused at pH 5,1. The main advantage of this pro= cedure was the higher yield of the

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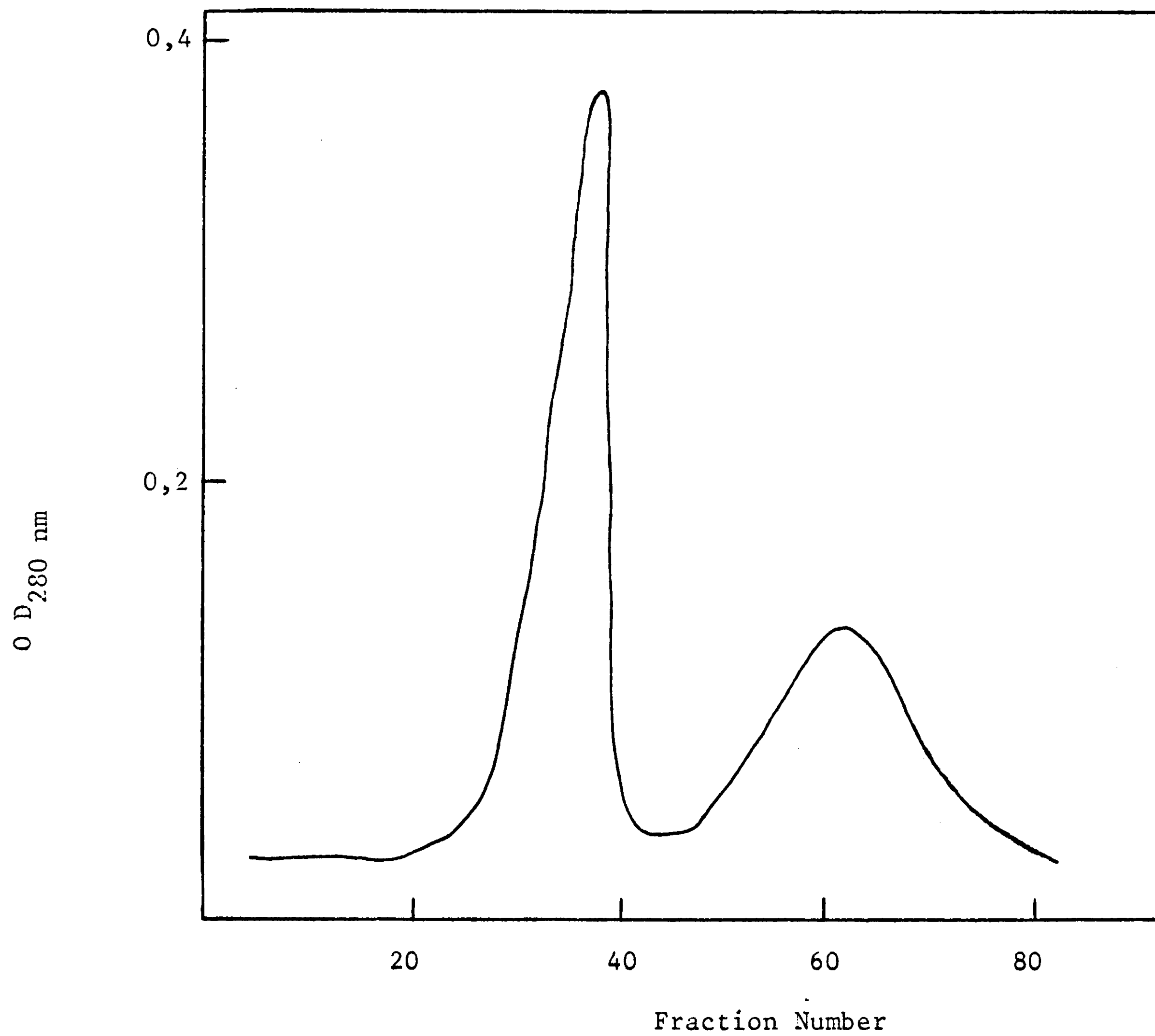

Fig 8 Chromatography on Sephadex G100 of the toxic fraction, obtained after isoelectric focusing and subsequent dialysis.

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toxic fraction (4 mg ml^{-1} crude salivary secretion). The isoelectric focusing pattern obtained by this method is shown in Fig 9. The fractions with isoelec= tric points of 5,1 and 4,8 are referred to as toxic and non-toxic components (or as toxin and non-toxin), respectively.

2.3.5 Homogeneity determinations

Isoelectric focusing employing poly= acrylamide gels (performed as described in 2.3.4) showed one zone only after the toxic component was investigated by this method. Furthermore, one peak was ob= tained after Sephadex G100 chromatography (See Fig 8). In addition, sedimentation equilibrium centrifugation at various sample concentrations indicated a homoge= neous preparation (Section 2.4.3). In addition amino acid sequence determinations showed that one protein only was present (Section 2.4.9).

2.4 CHARACTERIZATION OF THE PURIFIED TOXIC COMPONENT

2.4.1 Ultraviolet spectrum and nitrogen content

The ultraviolet absorption spectrum was obtained with a Beckman DK2A ratio re= cording Spectrophotometer employing 1 cm path length silica cells. The mass of the samples were determined on a Cahn electrobalance using a 0,1 mg range on the recorder. Samples were dissolved in 3 ml

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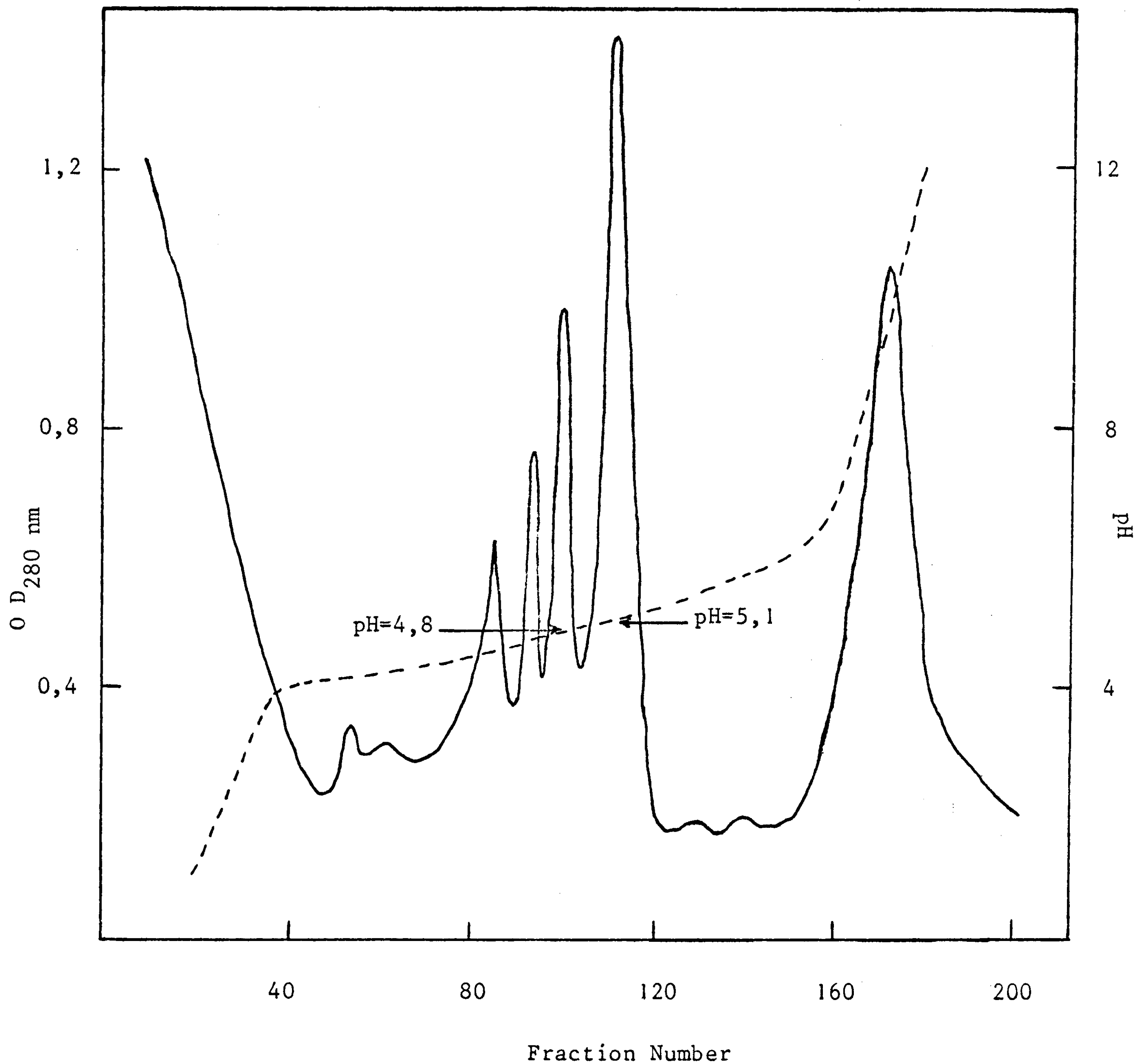

Fig 9 Isoelectric focusing of the toxic component obtained after Sephadex G100 chromatography; —— indicates absorption at 280 nm and --- pH measurements.

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0,08M NaCl; 0,02M Tris buffer at pH 7,9. The solvent was used as reference. The molecular extinction coefficient at this wavelength was $15{,}4 \times 10^{3}$ l mole^{-1} cm^{-1}. The spectrum is shown in Fig 10.
The nitrogen content of the toxic fraction was determined by the micro-Kjeldahl method (42).

2.4.2 Amino acid analysis and calculation of the minimum molecular mass.

Amino acid analyses were performed on acid hydrolyzates in a Beckman Model 120B amino acid analyzer. The mass of salt-free, lyophylized samples to be hydrolyzed were determined with a Cahn electro-balance. The mass of the samples was usually between 0,5 and 1,0 mg. Samples were transferred to 0,9 cm x 15 cm thick-walled glass tubes. Such a volume of 6N HCl (AR grade) was added so that the final concentration of sample in the acid was approximately 3 mg ml^{-1}.
The tube was inserted into a dry-ice ace= tone mixture. After the sample had frozen, the tube was evacuated by means of an oil pump to below 13,3 Pa. It was then with= drawn from the ice-bath while under vacuum and the frozen sample allowed to thaw slowly while the tube was shaken gently. Prompt momentary immersion of the tube in= to the ice-bath was necessary during this

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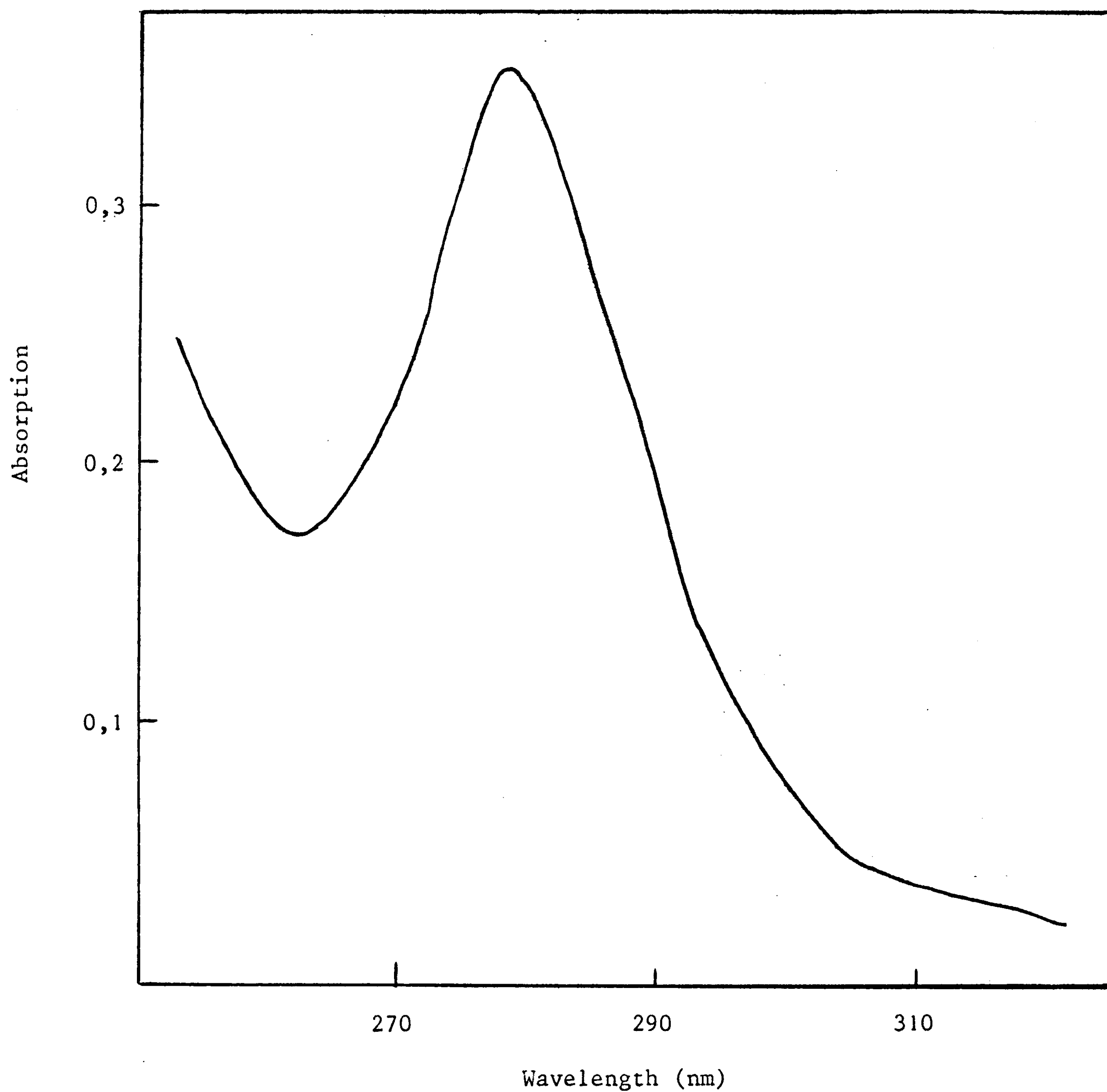

Fig 10 Ultraviolet absorption spectrum of the toxic component obtained after isoelectric focusing.

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process to avoid bubbles, formed in the sample, from rising too high up the tube. The thawing and refreezing of the sample was continued until bubble forma= tion had ceased, after which the sample was finally frozen and the tube sealed off while the vacuum was better than 13,3 Pa.

Hydrolysis was conducted at 110 ^{0}C in an oven for 20 hours. After the hydrolysis period the tube was cooled to room tem= perature and centrifuged at low speed in a bench top centrifuge and then opened. The HCl was removed at 40 ^{0}C under a steady stream of nitrogen in a fume hood. The dried sample was dissolved in 0,2N sodium citrate buffer at pH 2,2 and transferred quantitatively to a 5 ml volumetric flask and made to volume with the same buffer. A 1 ml aliquot was analyzed on the amino acid analyzer according to the conditions described in the Model 120B amino acid analyzer Instruction Manual.

Cysteine, together with cystine and methionine were determined as cysteic acid and methionine sulfone, respectively after performic acid oxidation at 50 ^{0}C. The oxidation was conducted as follows: Performic acid was prepared by adding 0,5 ml of 30% H_2O_2 to 4,5 ml of 90% formic acid at 50 ^{0}C (52). After 3 minutes, 50 µl was transferred

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to a hydrolysis tube containing approxi= mately 1 mg of protein. The reaction mixture was kept at 50 °C for 15 minutes after which the tube was placed in an ice mixture and the sample dried under a stream of nitrogen. Hydrolysis with 6N HCl and analysis of the sample was performed as described above.

The amino acid analyzer was calibrated by analyzing a synthetic mixture of the pure amino acids obtained from Beckman Intruments, Munich. The calibration was conducted each time fresh ninhydrin was prepared.

The tryptophan content was determined by the spectrophotometric method of Goodwin & Morton (45, 53). This method entails the measurement of optical densities of a solution of a protein in 0,1N NaOH at wavelengths of 280nm and 294,4nm and the substitution of these values in the equation :

$$\frac{M_{tyr}}{M_{trp}} = \frac{0,592(OD_{294,4}) - 0,263(OD_{280})}{0,263(OD_{280}) - 0,170(OD_{294,4})}$$

In this equation OD refers to optical den= sities measured at the wavelengths indi= cated by the subscripts and M_{tyr} and M_{trp} refer to the gram moles of tyrosine and tryptophan respectively, in one gram of protein.

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The results of the amino acid analysis are summarized in Table 7. In Table 8 the amino acid composition of the toxic and non-toxic components are compared. The amino acid data indicate that the toxic and non-toxic components contain compo= nents other than amino acids (See also Section 2.4.5). The minimal molecular mass of the protein portion of the toxic component, calculated by adjusting the amino acid yields to 100% recovery, is shown in Table 9.

2.4.3 - Molecular mass determination

The molecular mass of various isolated and chemically modified components were determined by sedimentation equilibrium centrifugation in a Beckman Model E analytical centrifuge equipped with an ultraviolet photoelectric scanner, mirror optics, multiplex system and schlieren and interference optics. The thermistors of the rotors were calibrated according to the method described in the Model E Instruction Manual. A rotor temperature of 20 °C was used in all the determinations. For molecular mass determinations at high sample concentrations (above 1 mg ml^{-1}), combined schlieren and interference optics were used and for lower concentrations the ultraviolet photoelectric scanner.

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TABLE 7 CALCULATION OF AMINO ACID COMPOSITION AND MOLECULAR MASS OF THE TOXIC COMPONENT

Amino Acid	gram residue in 100 g sample	Minimal molecular mass; amino acids	Residues per molecule	Calculated minimal molecular mass	Calculated residues per molecule	Residues to nearest integer
Lysine	7,68	1669	4	6676	3,8	4
Histidine	2,05	6693	1	6693	0,9	1
Arginine	2,68	5828	1	5828	1,1	1
Aspartic acid	8,39	1372	5	6860	4,6	5
Threonine	5,03	2010	3	6030	3,2	3
Serine	2,84	3067	2	6134	2,1	2
Glutamic acid	0,47	1363	5	6815	4,7	5
Proline	3,07	3098	2	6196	2,0	2
Glycine	4,53	1260	5	6300	5,0	5
Alanine	2,26	3146	2	6292	2,0	2
Valine	4,82	2056	3	6168	3,1	3
Isoleucine	2,11	5360	1	5360	1,2	1
Leucine	3,60	3142	2	6284	2,0	2
Tyrosine	5,35	3049	2	6098	2,1	2
Phenylalanine	2,34	6291	1	6291	1,0	1
½ Cystine *	2,75	3753	2	7506	1,7	2
Methionine *	1,42	9239	1	9239	0,7	1
Tryptophan**	1,67	11150	1	11150	0,6	1
TOTAL	72,06		43	6346 ***		43

* Determined as cysteic acid and methionine sulfone respectively after performic acid oxidation. ** Determined spectrophotometrically. *** Average value (methionine and tryptophan not included in the calculation).

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TABLE 8 COMPARISON OF THE AMINO ACID COMPOSITION OF TOXIC AND NON-TOXIC COMPONENTS

Amino acids	Toxic component		Non-toxic component	
	Gram residues per 100 g sample	Residues per molecule	Gram residues per 100 g sample	Residues per molecule
Lysine	7,68	4	7,96	4
Histidine	2,05	1	2,12	1
Arginine	2,68	1	2,91	1
Aspartic acid	8,39	5	8,51	5
Threonine	5,03	3	5,10	3
Serine	2,84	2	3,03	2
Glutamic acid	9,47	5	9,79	5
Proline	3,07	2	3,00	2
Glycine	4,53	5	3,80	4
Alanine	2,26	2	3,30	3
Valine	4,82	3	5,17	3
Isoleucine	2,11	1	1,70	1
Leucine	3,60	2	5,26	3
Tyrosine	5,35	2	4,90	2
Phenylalanine	2,34	1	2,16	1
½ Cystine ✦	2,75	2	3,20	2
Methionine ✦	1,42	1	2,17	1
Tryptophan✦'	1,67	1	2,29	1
TOTAL	72,06	43	76,37	44
Minimal molecular mass *f*	6346		6293	

✦ Determined as cysteic acid and methionine sulfone respectively after performic acid oxidation.

✦' Determined spectrophotometrically.

f Calculated from the amino acid composition (See Table 7).

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TABLE 9 CALCULATION OF THE MINIMAL MOLECULAR MASS OF THE PROTEIN PORTION OF THE TOXIC COMPONENT

Amino Acids	1 Gram resi= due in 100 gram sample	2 * Gram resi= due in 100 gram pro= tein	3 Minimal mo= lecular mass; amino acids *f*	4 Residues ** per mole= cule	5 Calculated minimal molecular mass
Lysine	7,68	10,48	1223	4	4892
Histidine	2,05	2,80	4900	1	4900
Arginine	2,68	3,66	4268	1	4268
Aspartic acid	8,39	11,44	1006	5	5030
Threonine	5,03	6,86	1474	3	4422
Serine	2,84	3,87	2250	2	4500
Glutamic acid	9,47	12,92	999	5	4995
Proline	3,07	4,19	2270	2	4540
Glycine	4,53	6,18	924	5	4620
Alanine	2,26	3,08	2308	2	4616
Valine	4,82	6,57	1508	3	4524
Isoleucine	2,11	2,88	3927	1	3927
Leucine	3,60	4,91	2303	2	4606
Tyrosine	5,35	7,30	2234	2	4468
Phenylalanine	2,34	3,19	4614	1	4614
½ Cystine	2,75	3,75	2752	2	5504
Methionine	1,42	1,94	6763	1	6763
Tryptophan	1,67	2,28	8168	1	8167
Ammonia	1,23	1,68			
TOTAL	73,29	99,98		43	4652 *'

* Values of column 1 corrected to 100% recovery.

f Calculated from values in column 2

** See Table 7

Note : For methionine, ½ cystine and tryptophan see footnote in Table 7

*' Average value (methionine and tryptophan not in= cluded in the calculation).

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In the determinations employing the combined schlieren and interference optics, the concentration distribution of the solute at equilibrium is recorded by both the interference and schlieren optics. The interference fringes indi= cate changes in concentration across the cell, while the schlieren pattern is used to determine the concentration gra= dient at corresponding positions (54). Both the schlieren and interference pat= terns for each photograph are superim= posed in this method. This is conve= niently accomplished by placing a mask, 1,5 mm in width over the schlieren light source in such a way that a narrow strip of light of the schlieren image is cut off. This allows a normal schlieren pattern to be recorded. An interference pattern is then photographed without moving the photographic plate. The fringe shifts are converted into equivalent schlieren areas by use of a conversion factor (C F), which is conve= niently determined with a filled Epon synthetic boundary cell and ribonuclease as sample. Both the schlieren and inter= ference patterns are recorded and the schlieren areas and fringe counts, calcu= lated with a Nippon Comparator, substitu=

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ted in the equation (55).

$C\ F = A \tan \theta / J$

In this equation θ is the phaseplate angle, A the area under the schlieren peak and J the fringe count across the boundary. The C F value of the instru= ment, used in all the determinations reported in this thesis, was found to be 0,11.

For molecular mass determinations, a 12 mm double sector cell with interference win= dow holder and sapphire windows was used. The sample, 0,12 ml was layered over 0,03 ml of FC-43 fluorocarbon oil (3M company) in the sample side of the cell. The solvent, (0,19 ml) was introduced in= to the solvent compartment. The cell was spun in an An-D rotor at 20 410 rpm for 70 minutes after which the rotor was decelerated to 13 410 rpm by reducing the voltage to the drive. The initial overspeeding of the rotor was used to shor= ten the transient time to equilibrium (56). The rotor was spun at the low speed for 17 hours during which period schlieren and interference photos were taken on metallographic plates. The phase plate angle was 60^0 and the exposure time 5 seconds for the schlieren and 40 seconds for the interference photographs.

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Photographic plates were developed with Kodak D19 developer for 10 minutes, rinsed for 30 seconds in Kodak SB5 stopbath and finally fixed with Kodak F5 fixer for 10 minutes. The plates were subsequently washed under running tap water for 20 minutes and dried at room temperature. Measurements of dc/dr (the concentration gradient, see equation below) at each radial position, r, determined from the fringe pattern, were made with the Nippon Comparator. A plot was made of $1/r(dc/dr)$ versus Δj, the fringe number counted from the solution meniscus. The numeri= cal value of the slope was subtituted in the equation :

$$M = \frac{RT}{(1-\upsilon\rho)\omega^2} \frac{d}{dc}\left(\frac{1}{r}\frac{dc}{dr}\right)$$

$$= \frac{RT}{(1-\upsilon\rho)\omega^2} \frac{\text{slope}}{\left(\frac{C\ F}{\tan\theta}\right)}$$

Where M is the molecular mass, ρ the so= lution density ($g\ cm^{-3}$), υ the partial specific volume ($cm^3\ g^{-1}$), R the univer= sal gas constant and r the radial dis= tance in cm corrected for the camera lens magnification.

For the lower concentrations the photo= electric scanner was used (57, 58). (This method was used for all molecular mass determinations when the scanner became available in this laboratory).

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The linearity of the photoelectric scan= ner response to variations in sample con= centrations in the cell was tested in the following way. Three double sector scan= ner cells with carbon centre pieces were filled with water in the reference side of the cells and three dilutions of an adenosine triphosphate stock solution in water introduced into the sample com= partments. The optical densities of the sample solutions, measured in a Beckman DK2A Spectrophotometer at 280nm & 270nm ranged form 0,18 to 0,57. The cells were placed into an An F rotor and spun at 15000 rpm. At this speed the optical densities were uniform throughout the cells. The multiplex system was used to trace the optical densities of each cell auto= matically, at 280nm and 270nm. A direct proportionality was obtained between the pen deflection and optical density (Fig 11).

The linearity of the scanner calibration stairsteps which provide a means by which recorder deflections for solutions in the cell may be converted to optical density units, was tested by plotting the recorder deflection of the stairsteps against op= tical density. For this test an optical density range between 0 and 1 was selected.

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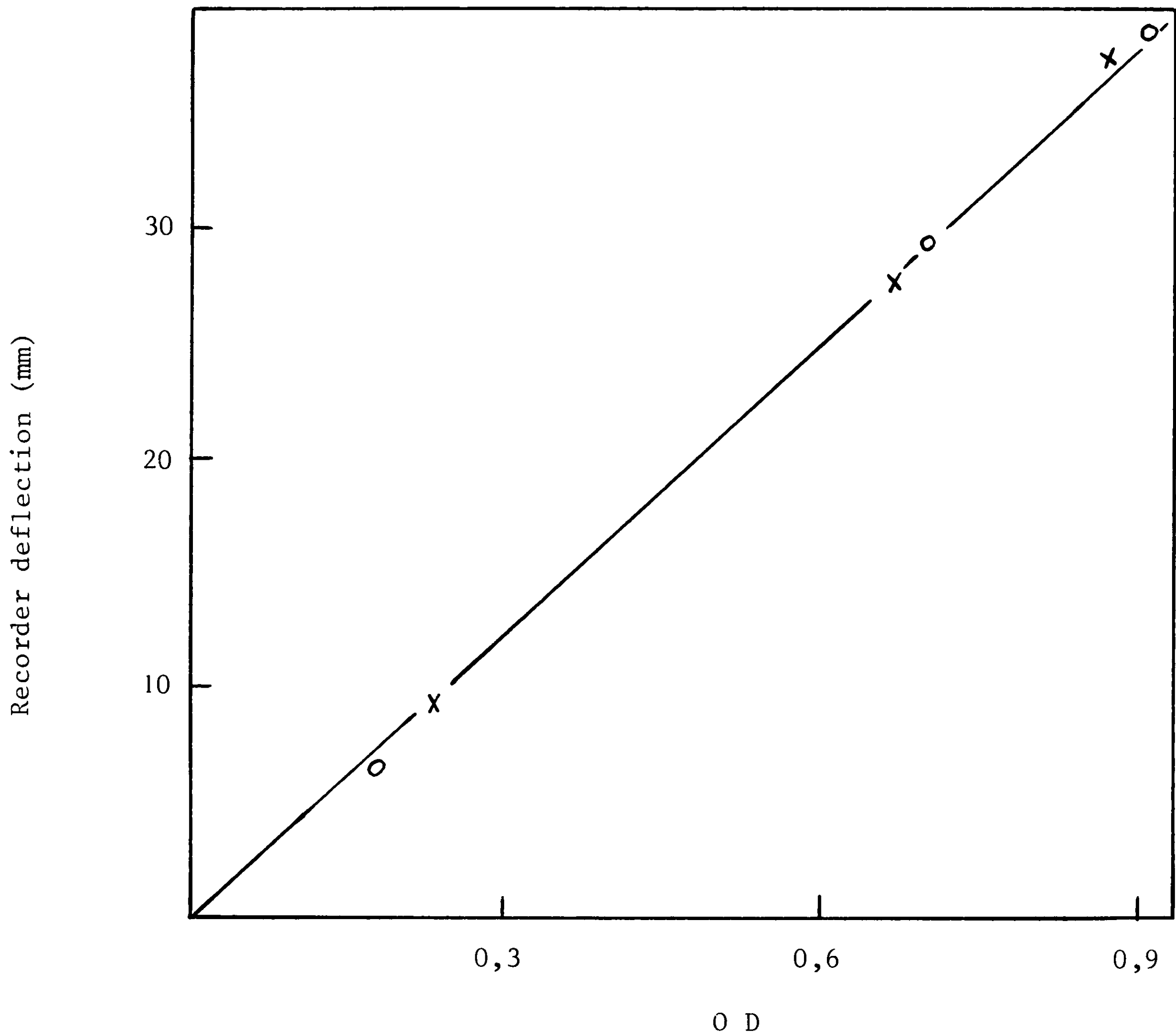

Fig 11 Linearity of the recording system of the photoelec= tric scanner as a function of optical density. The recorder deflection in mm is plotted on the ordinate as a function of optical density on the abscissa. Optical densities were recorded in a Beckman DK2A ratio recording spectrophotometer. Measurements were made with adenosine triphosphate at 280 nm (o) and at 270 nm (x).

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Five stairsteps were obtained, each representing 0,2 optical density units. The results showed a slight deviation from linearity.

For determinations of molecular mass with the photoelectric scanner, the scanner double sector cell was filled with solvent and sample solution as described for the combined schlieren and interference method. An initial overspeeding of the rotor was used before the rotor was spun at equili= brium speed. Scans were made manually during the run. Calibration of stair= steps made before each scan, were used to measure the optical density at radial positions, 5 mm apart, across that por= tion of the cell corresponding to the solution. Since the relationship of concentration of the samples and the optical densities (O D) were found to be linear in the concentration ranges used in the ultracentrifuge, O D values were not converted to corresponding values of concentration in further calculations.

A plot of log(O D) versus r^2 was made and the numerical value of the slope was substituted into the equation :

$$M = \frac{2RT}{(1-\upsilon\rho)\omega^2} \quad \frac{2,303\ d(\log O\ D\ )}{dr^2}$$

A typical plot of log (O D) versus r^2 is shown in Fig 12.

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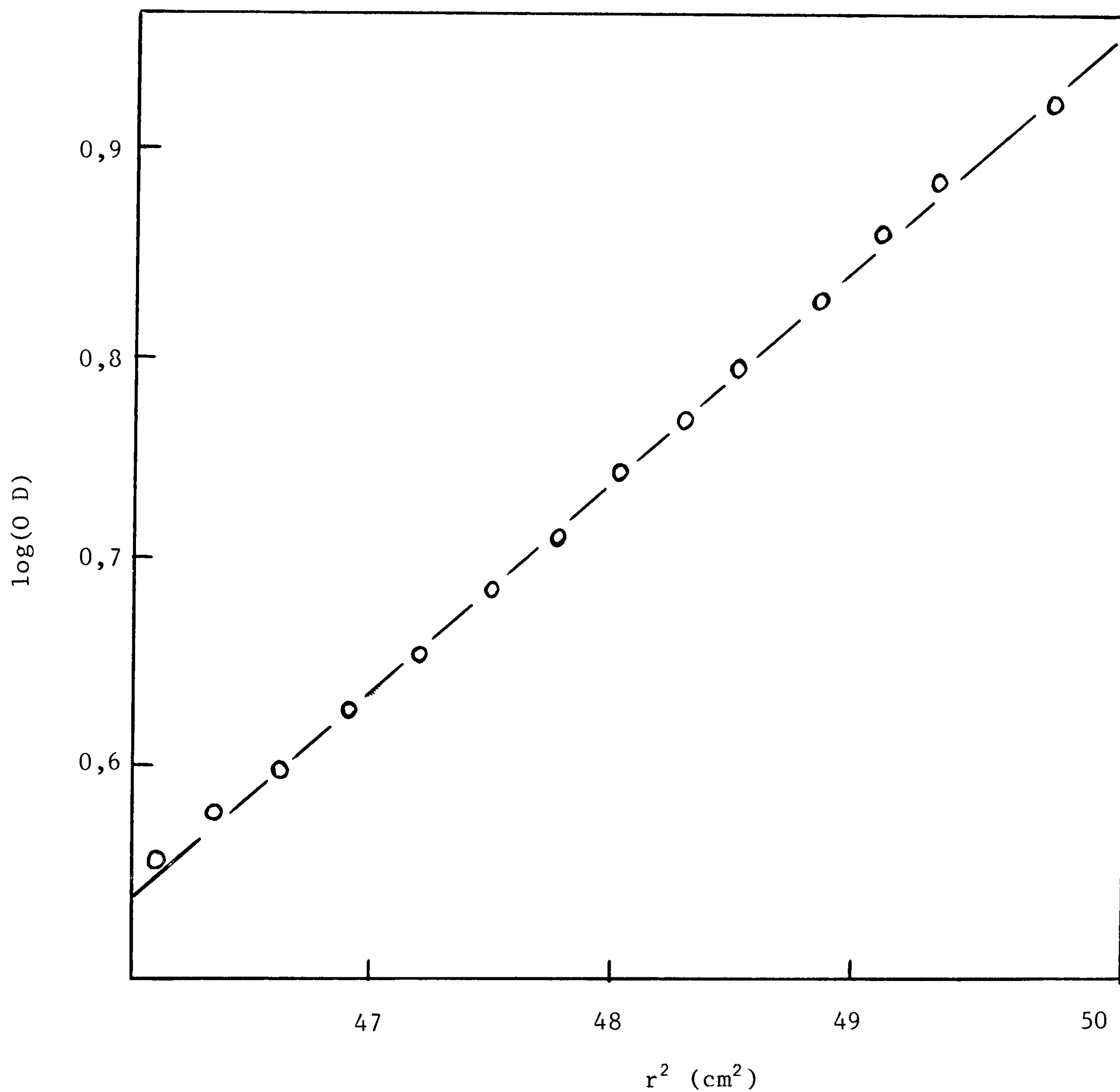

Fig 12 Typical plot of log(O D) versus r^2, used for molecular mass calculations by the sedimentation equilibrium method employ= ing the photoelectric scanner. The optical densities were determined from the calibration stairsteps.

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A summary of the molecular mass of the toxin, under various conditions op pH and concentration as well as the mole= cular mass of the chemically modified toxin, is presented in Table 10.

The dependence of the molecular mass of the toxin on concentration and pH is shown in Fig 13.

Some comment regarding the influence of rotor speed on equilibrium conditions is necessary. It was found during the course of the ultracentrifugal analysis that equilibrium conditions were reached over a wide range of rotor speeds, pro= vided the sample was homogeneous. Further= more, the molecular mass, calculated at these different equilibrium conditions showed little variation. This was clear= ly shown with ribonuclease (Fluka). The results of the molecular mass determinations obtained at different rotor speeds is shown in Table 11.

2.4.4 Determination of the partial specific volume

Determinations of the molecular mass of proteins by sedimentation equilibrium centrifugation require values of the partial specific volume, υ of the proteins. Accurate values of υ are necessary, since an error of 0,3% generally leads to an error of 1% in the calculated molecular mass (57).

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TABLE 10 SUMMARY OF THE MOLECULAR MASS OF THE TOXIN AND CHEMICALLY MODIFIED TOXIN UNDER VARIOUS CONDITIONS OF pH AND CONCENTRATION. THE SOLVENT IN ALL CASES WAS 0,02M Tris, 0,08M NaCl. EXCEPT WHERE OTHERWISE INDICATED ALL RESULTS WERE OBTAINED WITH THE PHOTO=ELECTRIC SCANNER

Sample	pH	Concen= tration mg / ml	molecu= lar mass	Comments
Toxin	7,9	0,09	9700	
Toxin	7,9	0,18	11600	
Toxin	7,9	0,42	14800	
Toxin	7,9	0,70	14800	
Toxin	7,9	5,0	15400	by combined schlieren, and inter= ference optics.
Toxin	4,0	0,41	22282	
Toxin	9,9	0,41	11982	
Toxin	7,9	0,42	11692	Represents sample at pH 9,9 after adjustment of pH to 7,9 with HCl.
Toxin treated with 0,1N NaOH	7,9	0,41	2422	See Section 2.4.6
Toxin treated with NaOH/ Na_2SO_3	7,9	0,41	4492	See Section 2.4.6
CM-Toxin	3	0,41	>20000	See Section 2.4.7
CM-Toxin	7,9	0,41	7680	See Section 2.4.7
CM-Toxin	9,9	0,42	hetero= geneous	See Section 2.4.7
CM-Toxin trea= ted with 0,1N NaOH/ Na_2SO_3	7,9	0,41	2778	See Section 2.4.6
CM-Toxin trea= ted with 0,1N NaOH	7,9	0,42	4183	See Section 2.4.6
Toxin treated with ICH_2COOH with no prior reduction	7,9	0,41	7800	See Section 2.4.7

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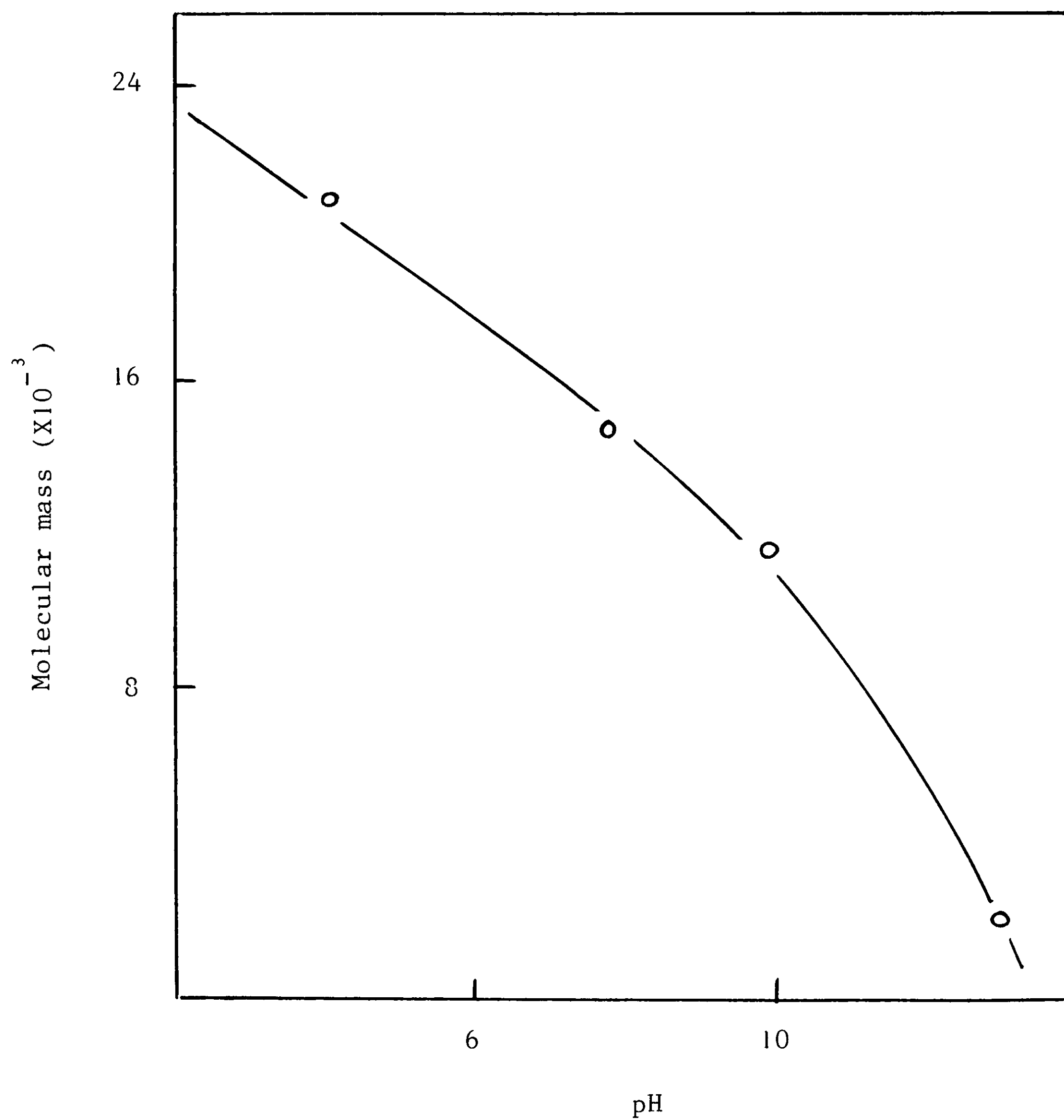

Fig 13(a) Dependence of the molecular mass of the toxic component on pH. The molecular mass was determined by the sedimentation equilibrium method.

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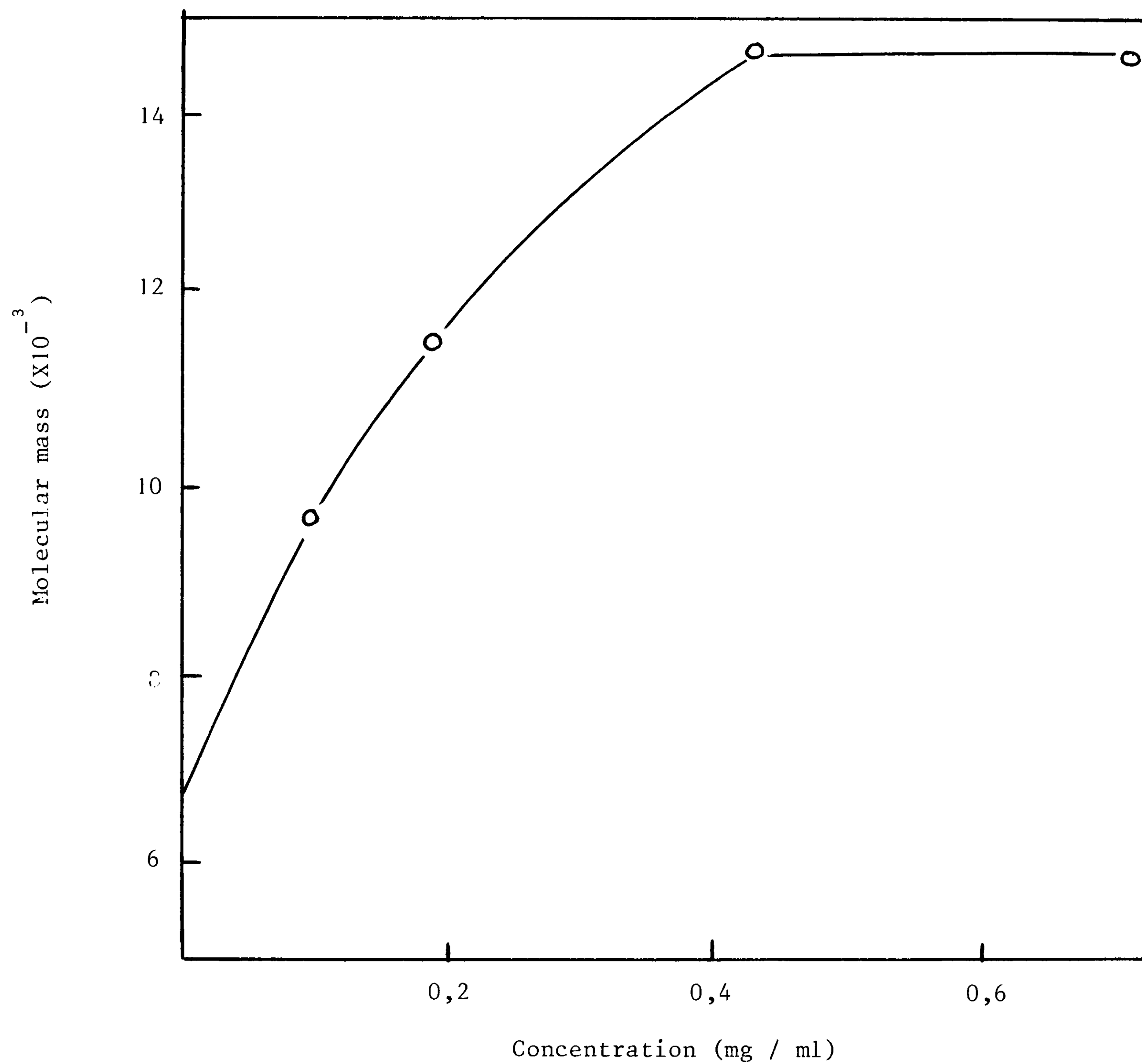

Fig 13(b) Dependence of the molecular mass of the toxic component on concentration. The molecular mass was determined by the sedimentation equilibrium method.

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TABLE 11 MOLECULAR MASS OF RIBONUCLEASE DETERMINED UNDER VARIOUS EQUILIBRIUM CONDITIONS

Rotorspeed rpm x 10^{-3}	OD_B / OD_M *	Molecular mass
12	1,48	13963
15	1,64	12209
20	2,45	13281
26	5,53	12873

* Ratio of optical densities at the bottom and meniscus of cell

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The classical methods for determining υ, require fairly large amounts of samples for accurate concentration and absolute density measurements. For differential density determinations the mass of a sinker in the sample solution and solvent may be determined with an electrobalance (59). Recently Kratky et al. (60) introduced a mechanical oscillator for the measure= ment of differential densities. Absolute densities may be determined by pycnometry (61), density gradient columns (62) or floats (63).

Partial specific volumes of proteins may also be obtained from the amino acid com= position (64). An assumption is made that the partial specific volume of the protein is an additive property of the partial specific volumes of the constitutive amino acids. The possible dependence of υ upon the three-dimensional structure of the protein is not taken account of. The partial specific volume of amino acid residues, calculated from their molal volumes and equivalent masses have been reported by Cohn & Edsall (64). The value of υ may be calculated from the equation,

$$\upsilon = \frac{\Sigma \upsilon_i W_i}{\Sigma W_i}$$

where W_i is the mass per cent of the i'th

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amino acid residue in the protein and
υ_i is the partial specific volume of this residue. The volume per cent of the i'th amino acid residue in the protein is
$\upsilon_i W_i$.

A method for the determination of υ of proteins which requires only small amounts of sample, of unknown concentration, was introduced by Edelstein and Schachman (65, 66). In this method, sedimentation equilibrium centrifugation is performed with proteins in solutions of H_2O and D_2O. Calculations are based on the sedimentation equilibrium equation (See Section 2.4.3), which may be presented as

$$M(1-\upsilon\rho_{H_2O}) = \frac{2RT}{\omega^2}\left(\frac{d\ \ln}{dr^2}\right)_{H_2O}$$

where the subscript indicates that the solvent is H_2O. When the solvent is D_2O, the molecular mass of the macro-molecule is increased as a result of deuterium exchange and the υ value is decreased by the same relative amount. Consequently, the sedimentation equilibrium equation becomes,

$$kM(1-\upsilon/_{k}\rho_{D_2O}) = \frac{2RT}{\omega^2}\left(\frac{d\ \ln c}{dr^2}\right)_{D_2O}$$

where k is the ratio of the molecular mass of the macro-molecule in deuterated solvent to that in the non-deuterated solvent. The two equations may be solved simultaneously for ...

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$$\upsilon = \frac{k-((d \ln c/dr^2)_{D_2O}/(d \ln c/dr^2)_{H_2O})}{\rho_{D_2O} - \rho_{H_2O}((d \ln c/dr^2)_{D_2O}/(d \ln c/dr^2)_{H_2O})}$$

The value of k may be estimated from a knowledge of the number of exchangeable hydrogen atoms of the protein (67). The value of k is considered to be relatively constant for almost all proteins since the exchangeable hydrogen atoms of proteins are principally the amide hydrogen atoms of the polypeptide chain (66).

In this investigation the partial specific volumes were determined from the amino acid composition (See Section 2.4.2) as described by Cohn & Edsall (64). The value was found to be 0,743 $cm^3\ g^{-1}$

The method of Edelstein and Schachman (66) was tested with RNase using H_2O and D_2O as solvents. However, it was found that the difference in $(d \ln c/dr^2)_{H_2O}$ and $(d \ln c/dr^2)_{D_2O}$ values were too small for accurate calculations. Consequently, this method was not used for the determi= nation of υ of the toxin. Greater accuracy may be obtained by using the denser D_2O^{18} as solvent (66, 68). This solvent was not used, however, due to its extremely high cost and limited availability.

2.4.5 Determination of Carbohydrates.

An early indication of the presence of carbohydrates in the toxic component was

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obtained from the amino acid deter= minations; the hydrolyzate developed a yellow to brown colour during hydroly= sis in 6N HCl at 100 ^{0}C. In addition, only approximately 72% of the toxic com= ponent could be accounted for by the constituting amino acids (See Section 2.4.2). The determination of carbo= hydrates present in glycoproteins pre= sents numerous problems as indicated in the short introductory note presen= ted below.

Sensitive colour reactions for carbo= hydrates present in glycoproteins are available, which do not require a prior hydrolysis of the carbohydrate component as a separate step (69). Most of these involve the reaction of the sugar with sulphuric acid to give a derivative of furfural or aromatic base. These derivatives or their reaction products formed by oxidation, reduction or con= densation in strong acids may produce coloured products with the sugars them= selves or with thiolic compounds, urea, phenols, aromatic amines and heterocyclic hydrocarbons. These reactions are possi= ble with poly- and oligosaccharides as well as with monosaccharides. In some cases, the sensitivity of the various classes of carbohydrates is of the same

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order of magnitude and in addition, the absorption maxima of the coloured products are very similar. Consequently, these colour reactions are particularly useful for the identification and estimation of total amounts of carbohydrates.
Colour reactions used for the estimation of total neutral sugars in glycoproteins include the anthrone (70), orcinol (71) and phenol-sulphuric acid reactions (69).
The anthrone reaction for total hexoses in the presence of proteins gives satis=factory results provided the amount of protein is not too large as compared to the hexose content (72). The only in=terfering amino acid residue is tryptophan which when present in large amounts may compete with anthrone for the furfural derivative (75).
Precise measurements of total hexoses in glycoproteins containing different sugars, is complicated by the fact that different colour yields are produced by various sugars. For example under reaction conditions described by Spiro (73), the approximate colour yields per mole as compared to galactose are, galactose 100, mannose 96, glucose 162 and fucose 60.
The orcinol reaction may be performed

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under specific conditions by which all classes of sugars react, making the method suitable for the determination of total carbohydrates (74). The sensitivity of the orcinol reaction is considerably greater at 420nm than at higher wavelengths. However, differences between different hexoses are substan= tially greater at the lower wavelength. Thus the ratio of the molar colour yields for galactose: mannose: fucose are 1,12: 1,00: 0,80.

A rapid and reliable procedure for the determination of total sugars has been described by Dubois et al. (76). The procedure utilizes phenol in the pre= sence of sulphuric acid as the organic colour developing agent. Apart from the simplicity, sensitivity and rapidity of the method, it offers the advantage that it is largely unaffected by the presence of proteins. In contrast to the anthrone and orcinol methods the heat required for colour development is provided by the exothermic reaction of sulphuric acid and water, thereby obviating the need for prolonged heat= ing of the reaction mixture. The coloured products of the reaction arising from hexoses, disaccharides, oligo-

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and polysaccharides including methylated derivatives possessing a free or poten= tially free reducing group, show an absorption maximum at 485-490nm. The pentoses and uronic acids produce pro= ducts with an absorption maximum at 480nm. The extinction coefficients vary among the different sugars (73). Amino sugars such as glucosamine and galactosa= mine do not react although they may be deaminated thereby rendering them reactive in the assay (77).

The above-mentioned colorimetric methods all suffer from the same disadvantages; the standards for the assays normally consist of free sugars and it is uncertain if glycosidically-linked sugars react in the same manner. Secondly, different sugars produce different colour yields and finally it is uncertain whether the entire colour yield can be attributed to the presence of sugars only. Neverthe= less, these methods are usefull for ini= tial detection of carbohydrates in glyco= proteins. They are also suited for moni= toring column effluents containing glyco= proteins.

The identification and quantitation of individual constituent carbohydrates pre= sent in glycoproteins generally involve

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hydrolytic, chromatographic and colori= metric techniques for their release, iden= tification and estimation. Automated ion exchange chromatography and gas-liquid chromatography for the separation and estimation of sugars have recently been improved and offer good sensitivity, re= solution and accuracy (78).

Almost all techniques used for quantita= tive estimations of the individual sugars, require the hydrolysis of the glycosidic linkages. Acid hydrolysis is at present almost invariably employed since glycosi= dic linkages are in general stable to alkali. In contrast with amino acids which are, with few exceptions, stable to conditions normally employed (6N HCl at 100 ^{0}C for 24 hours), the stabilities of monosaccharides found in glycoproteins vary greatly from one sugar type to another under the same conditions. The sugars are as a whole less stable than the amino acids with the exception of tryptophan.

When glycoproteins are considered, addition= al destruction may occur as the result of interaction of sugars with certain amino acids. Since these interactions involve amino acids, uncertainties arise in the determination of amino acids in glycopro= teins.

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The interaction between sugars and amino acids was first described by Maillard (79). The interaction leads to the formation of dark brown to black soluble or in= soluble humin material. The formation of brown material during acid hydrolysis of proteins may serve as a useful preliminary indication that a glycoprotein is present. The initial step in the sugar-amino acid interaction (Maillard reaction) is the for= mation of N-substituted glycosylamines. The unprotonated amino group of an amino acid and the anomeric hydroxyl are invol= ved in the reaction. The aldosylamine may undergo numerous reactions which eventually lead to the formation of colour= ed products.

Under strong acidic conditions and at high temperatures, the tendency for glycosyl= amine formation is low. Under these con= ditions, hexoses are degraded to 5-(hydroxy= methyl)-2-furfuraldehyde (HMF). Due to strong acidic conditions required for the decomposition of glucose, HMF formed from this sugar undergoes further de= gradation to levulinic and formic acids. The dicarbonyl and α,β-unsaturated carbonyl compounds formed, may react with the amino acids released from their peptide linkages, thus diminishing their yield.

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Although only the anionic form of the amino acid will react with carbonyl compounds, calculation shows that when at pH 0 for example, a 1M glycine so= lution still contains $4,5X10^{11}$ molecules of species with an unprotonated amino group (80). Since equilibrium will be re-established almost instantaneously on their removal, a substantial amount of amino acid may eventually be lost.
Further losses of amino acids may occur during the removal of the HCl after hydrolysis since favourable conditions such as pH, for interaction reactions prevail during the process.
Due to the destruction and interaction reactions of sugars described above and due to the differences in stabilities of the glycosidic bonds, generally suitable hydrolytic procedures are difficult to find. The problem is thus to find hydrolysis conditions under which all of a given sugar is released without being significantly destroyed. Some generalizations may be made. The hexosamines are the most resistant to destruction by acid and the glucosaminides show great resistance to acid hydrolysis (81). However, conditions which lead

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to hydrolysis of glycosidic linkages of glucosaminides also lead to the cleavage of acetamido bonds of N-acetylamino sugars (81). Glycosides of 2-deoxysugars are hydrolyzed up to 1000 times more readily than the corresponding derivatives of glucose (82). Ketosides are hydrolyzed more rapidly than aldosides (81, 82). Furthermore the rate of hydrolysis of glycosides in general depend upon the character of the aglycon, the size of the glycosylring and the anomeric configuration. (83). The differences in susceptibility of different glycosidic linkages towards acids hydrolysis may be readily explained if the mechanism of the hydrolysis is considered (81, 83).

Acid hydrolysis of most glycosidic bonds involve a preliminary protonation of the glycosidic oxygen atom to form the con= jugate acid followed by heterolysis of the exocyclic oxygen - C1 bond to give a carbonium-oxonium ion. Reaction with water leads to the formation of the protonated reducing sugar from which the reducing sugar is formed. Alternative mechanisms involving protonation of the ring oxygen have been proposed (84).

The extent of protonation of the glyco= sidic oxygen atom depends on the inductive

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effect of the substituent on C-2. This effect has been used to explain the dif= ferences in reactivity between the usual aldohexoses, 2-deoxyhexoses and the hexos= amines. Glycosides of N-acyl neuramic acid are readily hydrolysed under rela= tively mild conditions (0,025N-0,1N H_2SO_4 at 80 ^0C for 60 minutes). The reason is that the neuramic acids resemble 2-deoxy= sugars, the glycosides of which hydrolyze readily. Furthermore, the N-acyl neura= mic acids are ketosides which hydrolyze more rapidly than the aldosides.

Apart from acid hydrolysis of glycosidic linkages, enzymatic release of mono= saccharides of glycoproteins is possible. The use of enzymes offers many advantages. They can effect specific cleavages and may give information as to the anomeric configuration of the bond which is cleaved. Because of some interference of the protein chain of glycoproteins, these enzymes act more rapidly and completely on glycopeptides. Glycosidases which have proved to be of use in the study of the sugar components and their sequence in glycoproteins are neuraminidases, β-N-acetylglucosaminidase and almond emulsin (85). The latter is a crude enzyme preparation containing among others N-acetylglucosaminidase and

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mannosidase activity.

For the identification of the released sugar residues, after acid hydrolysis or enzymatic action, various methods may be employed. These include colorimetric and gas-liquid, paper, thin layer and ion exchange chromatographic methods (85).

Sialic acids and hexosamines may be de= termined by the following methods. The resorcinol reaction may be employed to determine both free and glycosidically bound sialic acids (86) and may thus be used directly on unhydrolyzed glyco= proteins. This method offers distinct advantages over the orcinol method (87). It is approximately 50% more sensitive. The orcinol method cannot distinquish between sialic acids and ketohexoses. The resorcinol method produces coloured products from naturally occurring carbo= hydrates with absorption spectra easily distinquishable from absorption spectra of the coloured products produced by sialic acids.

Hydrolysis of glycoproteins for the deter= mination of hexosamines is generally accom= plished in evacuated tubes at 100 °C in 3-4N HCl for 3 to 6 hours (81). After hydrolysis, glucosamine and galactosamine

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can be readily separated and determined by cation exchange chromatography in an amino acid analyzer.

In this investigation, total carbohydrates were determined by the phenol-sulphuric acid method (69). The toxic fraction (2,1 mg in 5 ml distilled water) was dia= lyzed for 16 hours against 1 l of distil= led water at 15 ^{0}C. The water was chang= ed after 4,8 and 12 hours. The sample was then freeze-dried (yield: 1,3 mg). Dialysis was performed in order to ascer= tain that traces of sucrose which might have remained after the isoelectric focu= sing procedure was removed. The freeze-dried sample was dissolved in 5 ml of water and aliquots, containing 0,24 to 0,45 mg of protein were transferred to 15x1 cm test tubes and water added to make a volume of 2 ml. To the sample was added 0,05 ml phenol reagent (80% phenol prepared by adding 20 g of water to 80 g of reagent grade phenol. The solution had a pale-yellow colour). After mixing in a vortex test tube mixer, 5 ml of concentrated H_2SO_4 (AR) was added raidly and after 30 minutes at room temperature the optical density was de= termined at 480nm in a Beckman DBG spec= trophotometer. The blank was prepared

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as described above with 2 ml of water instead of the sample solution.
A standard curve was obtained with glucose. For this purpose, determinations were made as described above using 10, 20, 30, 40 50, 60 and 90 µg glucose per tube. The curve of O D at 480nm *versus* µg glucose per tube was linear up to 50 µg of glucose per tube. The standard curve was employed to calculate the total carbohydrate content of the sample. The average value was found to be 11,3%.
Sialic acids were determined with the re= sorcinol method (86). The resorcinol-HCl reagent was prepared in the following way. To a mixture containing 80 ml of concentra= ted HCl and 0,25 ml of 0,1M $CuSO_4$ was added 10 ml of resorcinol stock solution (2 g resorcinol in 100 ml of distilled water) and the volume made to 100 ml of distilled water. The reagent was prepared at least 4 hours before use. A blank reagent which was used to correct for non-specific colour development, contained the HCl and $CuSO_4$ but no resorcinol.
The assays were performed in 18x150 mm Pyrex test tubes. The toxic component (0,5 to 1mg) was introduced into the tubes and dissolved in 2 ml of distilled water. A sialic acid stock solution

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containing 27,6 μg ml^{-1} water was prepared and aliquots containing 2,8; 5,5; 11,0; 13,3; 27,6; 41,4 and 55,2 μg were intro= duced into test tubes and the volumes made to 2 ml. The resorcinol-HCl reagent (2 ml) was added to the tubes as well as to two tubes containing 2 ml of distilled water. The blank reagent was added to two tubes containing 5,5 and 41,4 μg sialic acid and to a tube containing 0,7 mg of toxic component. All the tubes were capped with glass marbles and heated for 15 minutes in a boiling waterbath. They were then cooled in a waterbath (approxi= mately 15 ^{0}C) after which 5 ml of isoamyl alcohol was added. The coloured reaction products were extracted into the organic phase by mixing on a vortex mixer. The tubes were then placed in an ice bath for 15 minutes after which the samples were centrifuged to separate the phases. The organic phase was removed with an Oxford pipettor and the O D determined at 580nm. The sialic acid content of the toxic component was found to be 0,93%.

The hexosamine content of the toxic com= ponent was investigated with the amino acid analyzer (44, 81) after hydrolysis of up to 1,3 mg of sample with 4N HCl for 2 to 4 hours at 100 ^{0}C in evacuated tubes. Not even the slightest indication of the presence

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of glucosamine or galactosamine was ob= served.

2.4.6 An investigation into the nature of the carbohydrate-protein linkage

Several distinct types of glycopeptide bonds have been described (88). All bonds involve the C-1 of the internal monosaccharide of the carbohydrate chain and a functional group of an amino acid in the peptide chain. The types of linkages are :

(i) The glycosylamine bond involving N-acetylglucosamine and the amide of asparagine.

(ii) The O-glycosidic bond involving serine or threonine and N-acetyl= galactosamine, galactose, xylose or mannose.

(iii) The O-glycosidic bond between hydroxylysine and galactose

(iv) The O-glycosidic bond between hydroxyproline and arabinose

Since the toxic glycoprotein from the salivary secretion of _O. savignyi_ does not contain N-acetylgalactosamine, hydroxylysine or hydroxyproline, link= age types (i) (iii) and (iv) described above may be eliminated. It thus seems likely that the bond may be of the O-glycosidic type involving either serine

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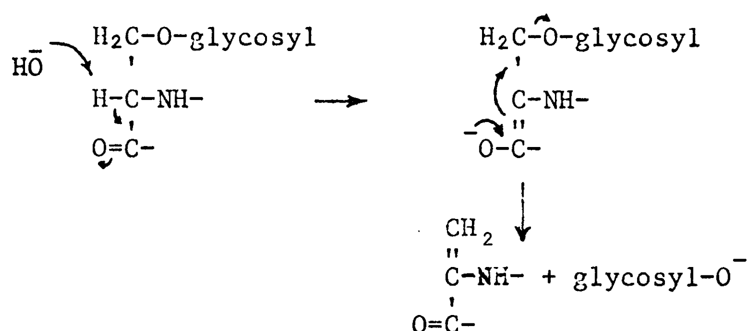
or threonine. Some characteristics of this bond are presented below.
Relatively mild alkali treatment of glyco= proteins containing carbohydrate units linked O-glycosidically to α-amino-β-hydroxy acid residues results in cleavage of the glycopeptide bond (89). Cleavage proceeds by mechanism of β-elimination of an alkoxide (the carbohydrate) (See Scheme 1)



Scheme 1 Mechanism of β-elimination

The effect of substituents in the amino acid residue on the reaction rate is noteworthy. For example Linstead (93) investigated the formation by alkali of crotonic acid from toluene-p-sulphonate (tosyl) and methane-sulphonate (mesyl) of ethyl-β-hydroxybutyrate. Quantita= tive yields of crotonic acid were ob= tained by alkali treatment under condi= tions which did not effect dehydration of the parent β-hydroxy ester. When the carboxyl group was not esterified, β-elimi= nation occurred only to a small degree. Riley (91)

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demonstrated the effect of an unsubsti=
tuted amino group on the β-elimination
process : He found that N-benzyloxycar=
bonyl-D,L-(di-O-phenylphospho)serine
ethyl ester is readily converted to α-
N-benzyloxycarbonyl-amino acrylic acid
and diphenylhydrogen phosphate. The
ester with a free amino group however,
resists β-elimination under the same
conditions. It is thus evident that
electron withdrawing groups facilitate
β-elimination since the acidity of the
α-hydrogen atom is increased. Neuberger
et al. (92) concluded that masking of
the amino and carboxyl groups in serine
and threonine, by engagement in peptide
bonds, is essential for β-elimination.
In the absence of reducing agent, β-
elimination of the carbohydrate moiety
is likely to undergo alkaline degra=
dation. For the indentification of the
sugar residue, the β-elimination should
be performed in the presence of sodium
borohydride. The sugar alditol formed
may be determined gaschromatographically.
(85, 93).

The amino acid involved in the bond may
be identified by observing any loss of
serine and threonine after acid hydrolysis
of the dehydropeptides. Carubelli et al.

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(94) followed the increase in absorbancy at 241nm upon alkali treatment of ovine submaxillary gland glycoproteins. The strong absorbancy exhibited by α-amino= acrylic acid and α-aminocrotonic acid derivatives in the lower U V region is well-known (91).
The α-aminoacrylic acid and α-amino= crotonic acid residues formed after the β-elimination reaction may be hydroge= nated to produce alanine and α-amino= butyric acid residues, respectively. Tanaka et al. (95) applied this procedure to bovine submaxillary glycoprotein. They found that with sodium borohydride as reductant, the reduction of dehydro= alanine was equal to the loss of serine. However, only 14,7% of the lost threonine was recovered as α-aminobutyric acid. This low yield may be partially explained by the results obtained by Adams (96) who found that mild alkali treatment of a glycoprotein isolated from human colloid breast carcinoma resulted in a conversion of the produced α-aminocrotonic acid residues to α-oxobutyric acid and glycine after acid hydrolysis. Bergmann and Grafe (106) have clearly shown that dehydropep= tides easily undergo hydrolysis under mild= ly acid or basic conditions. For example,

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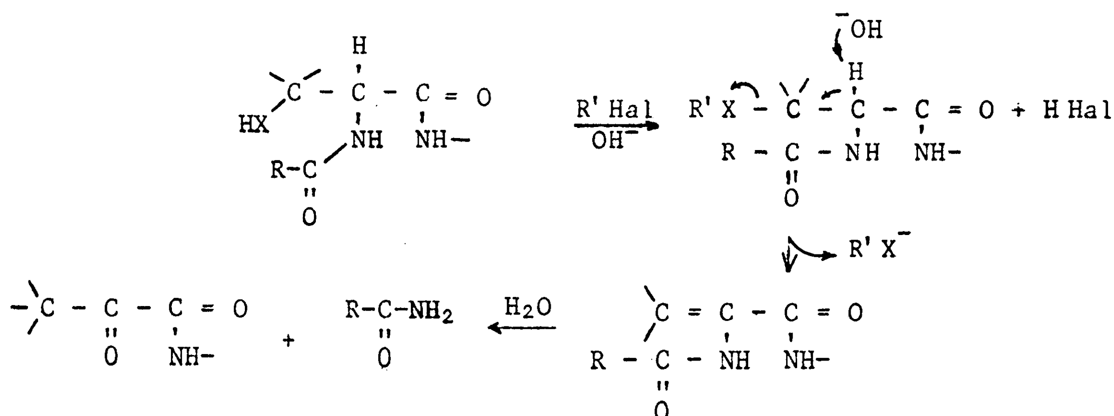
they found that α-acetaminoacrylic acid was degraded to pyruvic acid in the pre= sence of dilute hydrochloric acid and that the peptide bond of glycyldehydro= alanine was split by mild alkali treat= ment. The instability of dehydropeptides was also shown by Patchornik et al. (98) who developed a method for the selective cleavage of peptides at threonyl, seryl and cysteinyl residues. The approach is summarized in Scheme 2 (99). The β-hydroxyl of serine or threonine (X=O) or the β-sulfhydryl group of cysteine (X=S) is converted into a derivative OR' or SR'. The substituent R' is selected so as to make ⁻OR' or ⁻SR' a good leaving group in the subsequent β-elimination.



Scheme 2. Selective cleavage at threonyl, seryl or cysteinyl residues.

The α-oxobutyric acid or pyruvic acid may be determined enzymatically or by conden= sation with 2,4-diphenylhydrazine (100). Attention should be drawn to yet another

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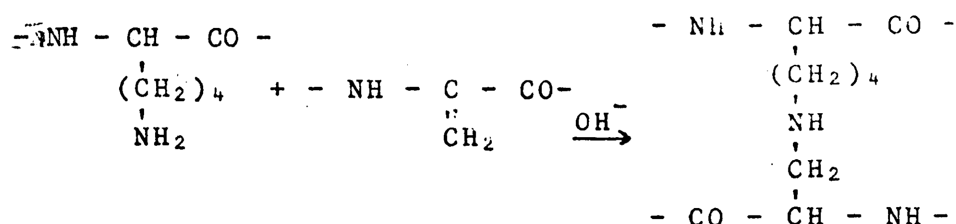
reaction involving dehydroamino acid re= sidues. This was observed in alkali trea= ted proteins in which cystinylpeptides where cleaved via dehydroalanine formation (101). Bohak (102) reported the formation of N-(DL-2-amino-2-carboxyethyl)-L-lysine or lysinoalanine during the alkaline treatment of some proteins as a result of the addition of the ε-amino group of a lysyl residues to the double bond of a dehydroalanyl residue (Scheme 3).



Scheme 3. Addition of a lysyl residue to the double bond of a dehydroalanyl residue.

Lysinoalanine emerges just before lysine during normal amino acid analysis pro= cedures (102). It seems that in all likelihood, lysinoalanine is readily formed upon alkaline treatment when lysine residues are adjacant or in close proximity to cystine, suitable O-substituted serine and threonine or suitable S-substituted cystein residues. A procedure employed by Harbon et al. (100) for the identification of the α-hydroxy-β-amino acid linked to the carbo= hydrate moiety of glycoproteins

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involves mild alkaline treatment in the presence of sulfite. Formation of sul= fonyl derivatives from the dehydroamino acid residues, cysteic acid from dehydro= alanine and α-amino-β-sulfonylbutyric acid from α-aminocrotonic acid serve as evidence for the involvement of serine and threonine, respectively, in the glyco= peptide bond. Cysteic acid and α-amino-β-sulfonylbutyric acid cannot be resolved by normal amino acid analysis. Separation may readily be achieved by employing a Dowex 1-X8 anion exhange resin (85).

The alkaline-sulfite treatment should be performed with glycoproteins in which all the half-cystine residues have been oxidized to cysteic acid, since variable conversion of cystine to cysteic acid may occur (85). In certain cases sulfonated amino sugars may form during alkaline-sulfite treatment of glycoproteins.

Weber & Winzler (103) reported the forma= tion of 3-sulfonylhexosamine from C-3 sub= stituted glycosidically linked hexosamine. This compound emerges sligthly behind the sulfonyl amino acids during amino acid analysis.

Apart from chemical cleavage of the O-glycosidic linkage to serine and threonine, enzymatic cleavage may be employed.

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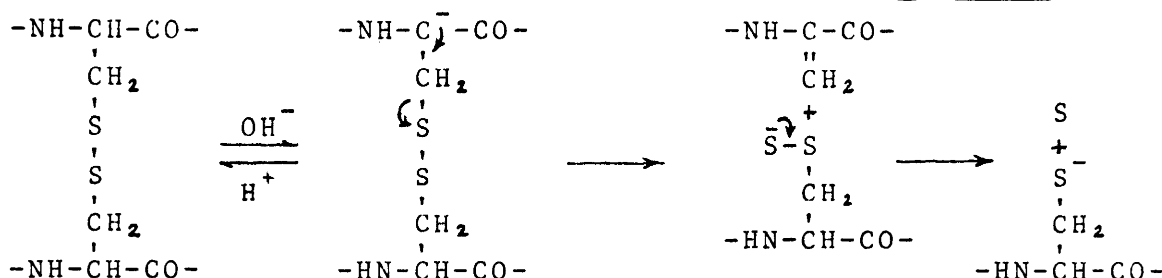
Weissmann et al. (104) and Buddecke & Werries (105) have isolated from ox liver and ox spleen, respectively, a highly purified N-acetyl-β-D-glucosaminidase (E.C.3.2.1.30). This enzyme was shown to act on numerous sub= strates including aryl glucosides of N-acetyl= glucosamine and N-acetylgalactosamine and on phenyl-N-acetyl-β-D-glucosaminide. Buddecke and Schneider (106) have shown that O-seryl glucosides are likewise cleaved by this en= zyme. They synthesized 1-O-(L-seryl-amide hydrochloride)-N-acetyl-β-D-glucosaminide which was hydrolyzed by the enzyme.

In this investigation an increase in absor= bancy at 241nm was observed after treatment of the toxic component with 0,5N NaOH at room temperature. The increase in absorbancy was recorded automatically with a Beckman DK2A ratio recording spectrophotometer with a sample containing 0,134 μmol in 3 ml of 0,5N NaOH. The pen speed was 2 cm min^{-1}. (Fig 14). Since the recording was conducted with a sample with intact disulfide bonds, 0,134 μmol dehydro= alanine is to be inferred as the result of alka= li cleavage of these bonds. (Scheme 4).



Scheme 4. Formation of a dehydroalanyl residue from cystinyl residues.

If it is assumed that the molecular extinction

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coefficient of the dehydroalanine residue formed during the alkali treatment is the same as that for α-N-benzyloxy=carbonyl-amino acrylic acid (ε=5300) (91), the absorbancy increase shown in Fig. 14 represents the formation of 0,16 μmol of dehydroalanine as early as after the first 11 minutes of the re=action. This indicates some additional source of a dehydroalanine residue (or an α-aminocrotonylic acid residue). An additional source may be serine or threo=nine residues involved in glycopeptide bonds.

To establish if these α-amino-β-hydroxy acids are indeed involved in glycopeptide bonds the toxic component was treated with alkali and subsequently hydrolyzed with acid. An amino acid analysis was then performed to reveal if any destruc=tion of threonine or serine residues had occurred. The experiment was performed in a manner similar to that published by Anderson (107). The toxic component, (1,2 mg) was dissolved in 0,2 ml of 0,5N NaOH in a hydrolysis tube (0,9 x 15 cm), flushed with nitrogen, stoppered and left at 5 ^{0}C for 48 hours. After this period, 0,2 ml of concentrated hydrochloric acid was added and the tube sealed under vacuum. Hydrolysis was carried out at 110 ^{0}C for 20 hours. A control sample (1,2 mg) was hydrolyzed with a 0,4 ml mixture

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TABEL S 3 GEDEELTELIKE AMINOSUUR VOLGORDE VAN DIE TOKSIESE EN NAVERWANTE NIE-TOKSIESE KOMPONENT, BEPAAL DEUR DIE METODE VAN EDMAN (S 24)

		1 ... 10
Toksiese komponent	*(TK)*	*Gly-CMCys-Pro-Pro-Gly-Val-Pro-Thr-Arg-Ala-*
Nie-toksiese komponent	(NTK)	Asp-CMCys-Pro-Pro-Thr-Lys-Pro-Thr-Arg-Ala-
		11 ... 20
	(TK)	*Tyr——Val-Ala-Phe-Val-Glu-Gly- - -Gly-Ala-*
	(NTK)	Tyr——Val-Ala-Phe- - -Glu-Gly-Glu- - -Ala-
		21 ... 30
	(TK)	*Tyr——Leu-Ile-Val-Val-Thr-Leu- - -Leu- - -*
	(NTK)	Tyr——Leu-Ile-Val-Val-Ser- - -Asp-Leu- - -
		31
	(TK)	*Ala——Leu-*
	(NTK)	Ala-

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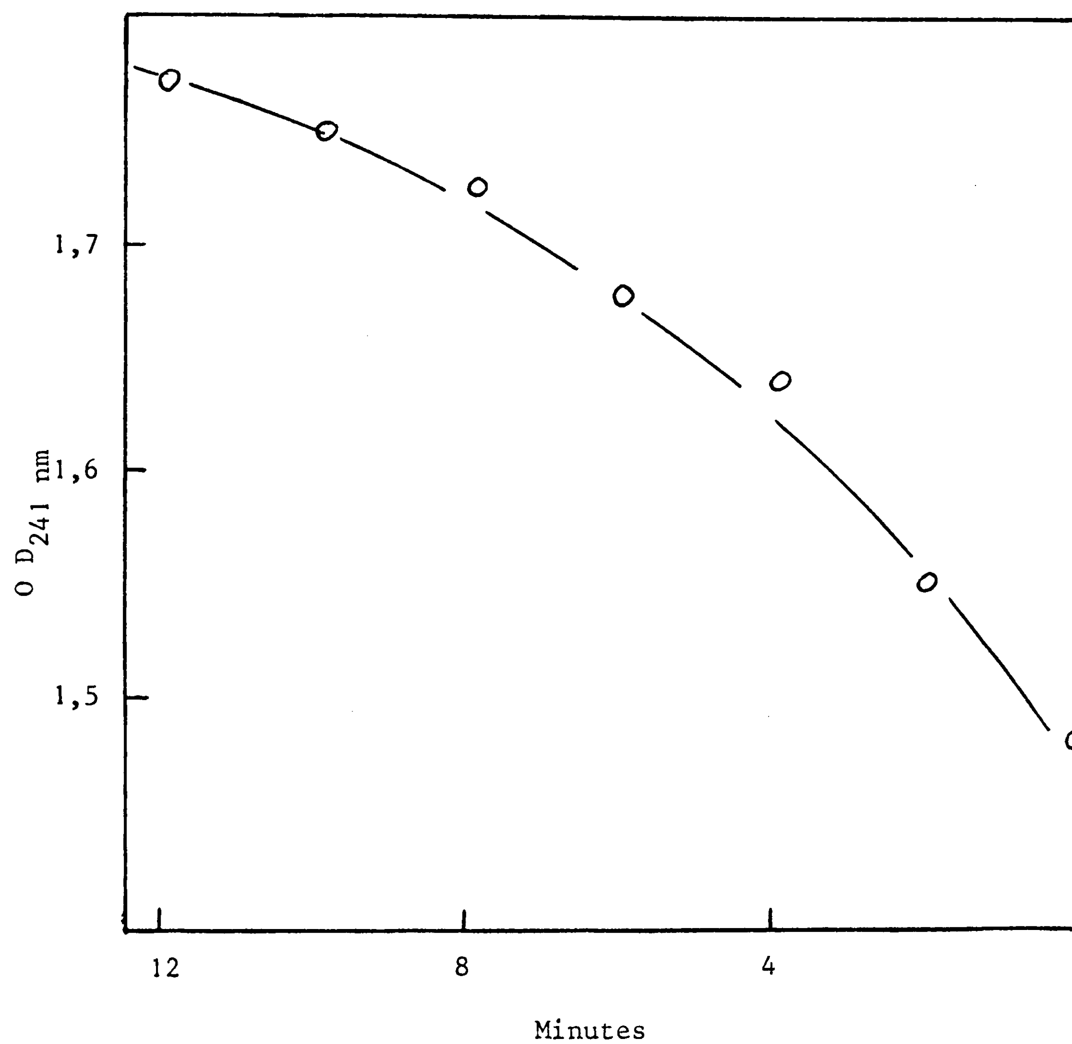

Fig 14 Change in absorption at 241 nm with time upon treatment of the toxic component with 0,5N NaOH at room temperature.

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consisting of equal parts of concentrated HCl and 0,5 N NaOH. Amino acid analysis was performed as described in 2.4.2.
The results revealed that 30 μmol of threonine per one gram of protein was lost as a result of the alkali treatment.
In addition to the alkaline treatment described above, the carboxymethyl toxic component (See Section 2.4.7) was subjected to alkaline sulfite treatment.
The carboxymethylated toxic component (CM-toxic component or CM-toxin) was chosen since variable conversion of cystine to cysteic acid occurs during alkaline sulfite treatment (85). It was shown that S-carboxymethylcysteine, submitted to alkaline sulfite treatment, as described below for the carboxymethyl toxic component, was stable under these conditions (no conversion to cysteic acid occurred). It was thus expected that the appearance of a peak at the cysteic acid position in the amino acid chromato= gram after acid hydrolysis of alkaline sulfite treated CM-toxin would be the re= sult of a nucleophylic addition of sulfite after β-elimination had occurred.
The procedure employed for the alkaline sulfite treatment was as follows: the CM-toxic component (1,2 mg) was dissolved

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in 0,2 ml of 0,1N NaOH containing 0,5M Na_2SO_3 in a 0,9x15 cm hydrolysis tube, stoppered and incubated at 37 °C for 60 hours. After this period, 25 µl was withdrawn with a microsyringe for ultracentrifugal analysis (See Section 2.4.3) To the remaining reaction mixture was added 30 µl of 6 N HCl and the mixture taken to dryness under nitro= gen. To the dried sample 0,3 ml of 6N HCl was added and the tube sealed un= der vacuum. Amino acid analysis was performed as described in Section 2.4.2. The results, summarized in Table 12 clearly show that the total ½ cystine, calculated from the cysteic acid and S-carboxymethylcysteine content is higher for the alkaline sulfite treated CM-toxin than the ½ cystine calculated from the cysteic acid content of performic acid oxidized toxin. In addition, the results show that a destruction of threonine occurs during the alkaline sulfite treatment. Furthermore, it is obvious that in contrast to the stabili= ty of free S-carboxymethylcysteine during alkaline sulfite treatment the S-carboxy= methylcysteine residues in the intact protein are almost totally converted to cysteic acid.

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TABLE 12 COMPARISON OF THE AMINO ACID COMPOSITION OF THE TOXIC COMPONENT AND ALKALINE-SULFITE TREATED CM-TOXIC COMPONENT

Amino Acids	Toxic fraction. Gram residues in 100 g sample	Alkaline-sulfite treated CM-toxic component. Gram resi= dues in 100 g sample
Lysine	7,68	7,26
Histidine	2,05	1,86
Arginine	2,68	2,54
Aspartic acid	8,39	8,62
Threonine	5,03	4,08
Serine	2,84	2,66
Glutamic acid	9,47	9,27
Proline	3,07	2,87
Glycine	4,53	4,32
Alanine	2,26	2,56
Valine	4,82	4,79
Isoleucine	2,11	2,07
Leucine	3,60	3,82
Tyrosine	5,35	5,28
Phenylalanine	2,34	2,35
½ Cystine	2,75✚	3,25✚'
Methionine	1,42✚	1,31✚
Tryptophan	1,67*f*	not determined

✚ Determined as cysteic acid and methionine sulfone respectively after performic acid oxidation.

✚' Determined as the sum of cysteic acid (2,88 g / 100 g sample) and CM-cysteine (0,37 g / 100 g sample)

f Determined spectrophotometrically.

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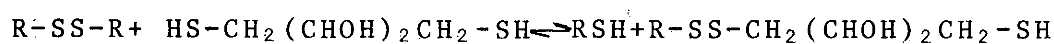
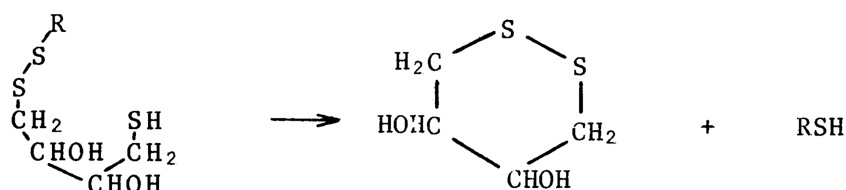
2.4.7 Reduction and S-Carboxymethylation

A procudure similar to that described by Crestfield *et al*.(108) was employed. Dithiothreitol (DTT) was used as reduc= tant (109). DTT has several advantages over the widely used reductant, β-mer= captoethanol. Dithiothreitol is oxidized to the cylic disulphide during the reduction of disulphides and this displaces the equilibrium to the right (Scheme 5).





Scheme 5. Reaction of disulphides (R-SS-R) with dithiothreitol

In addition to this favourable effect of DTT on the equilibrium, DTT possesses other convenient properties. Both the reduced and oxidized forms are soluble in water and aqueous solutions of the re= duced form have little odour and are re= markably stable to air oxidation. More= over, DTT has a low redox potential (-0,33 Volt at pH 7).

Reduction of proteins by thiols is com= monly performed in the presence of EDTA (oxidation of thiol groups are catalyzed

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by traces of heavy metal cations), 8M urea or 5M guanidinium chloride to render all groups accessible for reaction. Due to difficulties encountered in the purification of commercially available guanidinium salts, urea has most fre=quently been used. Urea solutions may give rise to cyanate ions on standing and it is imperative to use freshly prepared solutions to avoid cyanate modification of the protein. Cyanate is capable of reaction with amino, sulfhydryl, carboxyl, phenolic hydroxyl, imidazole and phosphate groups in proteins (110). The use of amine buffers for pH control, for example Tris, or methylamine results in the removal of any traces of cyanate. Preferential reaction of cyanate with the amine at concentrations in which these substances are normally used almost eliminates re=action with proteins. The reduced protein may be isolated if relatively strong acidic conditions, which avoids reoxidation of the sulfhydryl group, are maintained during the isolation. It is advantageous, however, to block the sulfhydryl groups, preferably direct=ly after the reduction reaction. Suit=able blocking reagents should show re=

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latively large differences in reacti= vity towards sulfhydryl groups in compa= rison to other nucleophilic amino acid side chains. Thus acrylonitrile which forms the S-cyanoethyl derivative of cysteine and ethylenimine which forms the S-amino-ethyl derivative are suitable blocking reagents. The most extensively used blocking reagent is iodoacetic acid. Excess iodoacetic acid is to be avoided to minimize modification of other amino acids. When the carboxymethylation is performed without prior isolation of reduced protein, the excess reducing agent must be taken into account. Consequently, the amount of iodoacetic acid required represents a large excess over the quantity needed to block the protein sulfhydryl groups. It is there= fore recommended to add slightly less on a molar basis of the blocking reagent than the theoretical required quantity (111).

Amino acids other than cysteine which may be modified by excess iodoacetic acid are methionine, histidine, lysine and tyrosine.

It should be borne in mind that iodide ions are formed during the carboxymethyla= tion reaction. The light-catalyzed

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oxidation of iodide to iodine should be prevented by performing the carboxy=methylation and subsequent removal of reagents and byproducts in the dark. Iodine may oxidize methionine to the sulf=oxide and form substitution products with tyrosine, histidine and tryptophan resi=dues. The extent of conversion of ½ cystine residues to S-carboxymethylcysteine may be determined by amino acid analysis. Furthermore the extent of side reactions such as sulfonium salt or sulfoxide for=mation from methionine and carboymethyla=tion of histidine and lysine may also be revealed by this procedure. Sulfonium salts are indicated by the presence of homoserine and homoserine lactone. Modi=fications of histidine and lysine are indicated by the presence of 3-carboxy=methylhistidine, 1-carboxymethylhistidine, ε-carboxymethyllysine and ε-dicarboxy=methyllysine. Since methionine sulfoxide is converted to methionine during acid hydrolysis (112), alkaline hydrolysis is required to establish the presence of this compound (113).

Reduction and carboxymethylation of the toxic and non-toxic components was per=formed in the following manner : between 0,5 and 1,5 μmol of protein (assuming a

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molecular mass of 15000) was used, a 100 fold excess of DTT and 6% less iodo=acetic acid than theoretically required (111).

The protein was introduced into a 5 ml screw-cap vial and 1,8 g of crystalline urea was added. The protein and urea were dissolved in a solution containing 0,15 ml of 5% EDTA solution and 1,5 ml of 14,4M Tris; pH 8,6 buffer. The solu=tion was thoroughly flushed with high purity nitrogen. The required amount of DTT was added and made up to a 3,75 ml mark with water. A 8M urea solution con=taining 0,2% EDTA was used to fill the vial completely. After flushing with nitrogen, the vial was tightly closed. After 4 hours at 25 ^{0}C the contents was transferred to a 10 ml conical flask and flushed with nitrogen, covered with alumi=nium foil to exclude light and 0,5 ml 1N NaOH containing the required amount of iodoacetic acid added. After 15 minutes at room temperature the solution was applied to a 2,5 x 40 cm Sephadex G 25 column which was equilibrated with 0,2N acetic acid. The column and inlet lines to the column were wrapped with aluminium foil. The eluant was 0,2N acetic acid and the flow rate 20 ml h^{-1}.

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Fractions of 2,6 ml were collected. The effluent was monitored spectro= photometrically at 280nm and with a Thomas Serfass conductance bridge with a type K-1X25 conductivity cell. A typical elution pattern is shown in Fig 15. The fractions containing the protein were combined and lyophilized. The recovery of the protein was usually in excess of 85%.

The extent of the reduction and S-carboxymethylation reaction as well as the specificity of the reaction was ascertained by amino acid analysis. Since traces of oxygen affects the re= covery of S-carboxymethylcysteine, parti= cular attention was given to complete deaeration of the hydrolysis mixture prior to heating. The amino acid elution diagram revealed that an essentially quantitative conversion of cysteine to S-carboxymethylcysteine had occurred and that no other amino acids were modi= fied.

Carboxymethylation of the toxic component under conditions described above but in the absence of DTT was conducted to determine if free sulfhydryl groups were present. Amino acid analysis of the toxic component treated in this way showed

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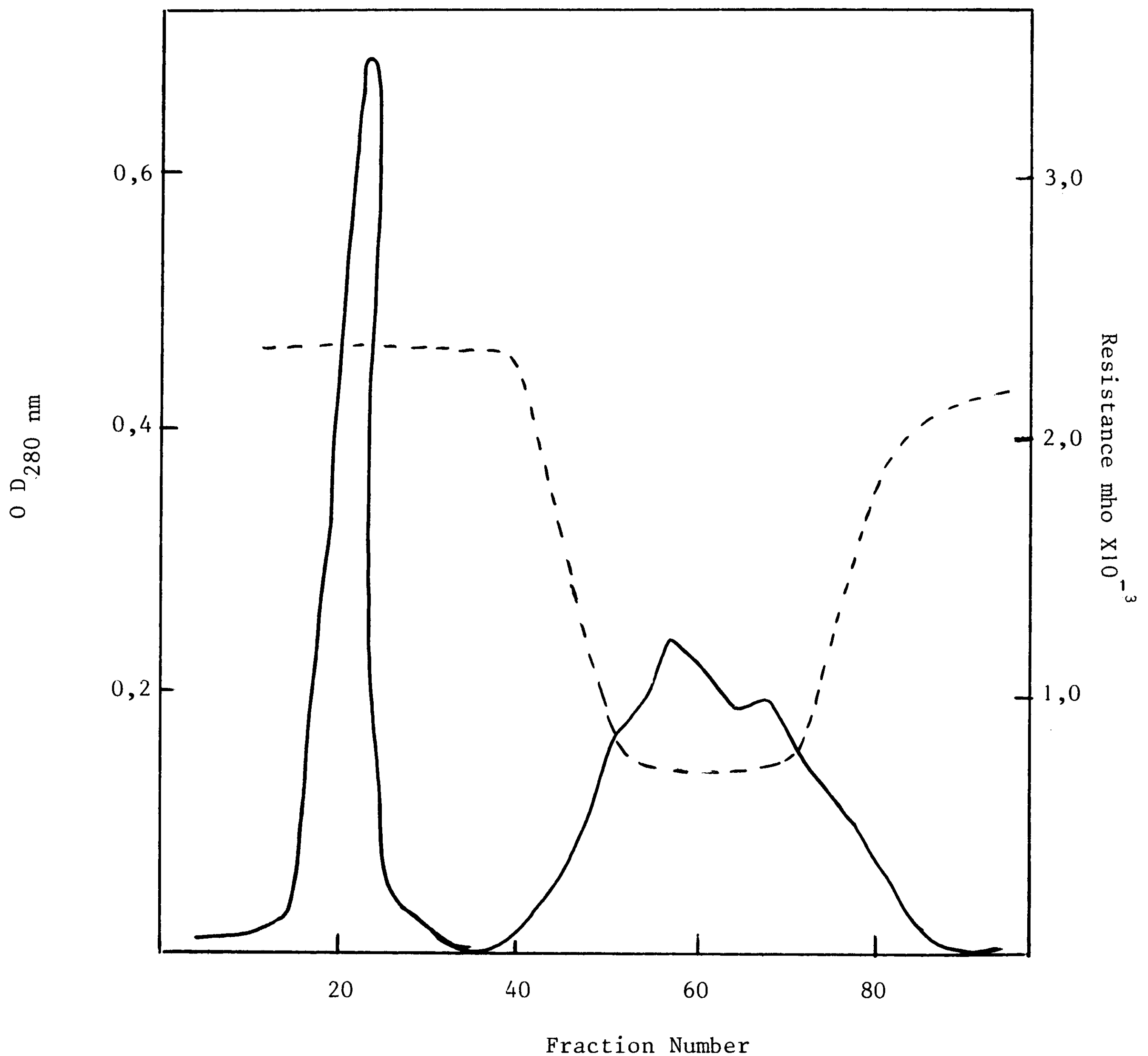

Fig 15 Removal of excess reactants and byproducts after S-carboxymethylation of the toxic component. Column : Sephadex G25 and 0,2N acetic acid as eluant. The resistance of the effluent is represented by the dashed line.

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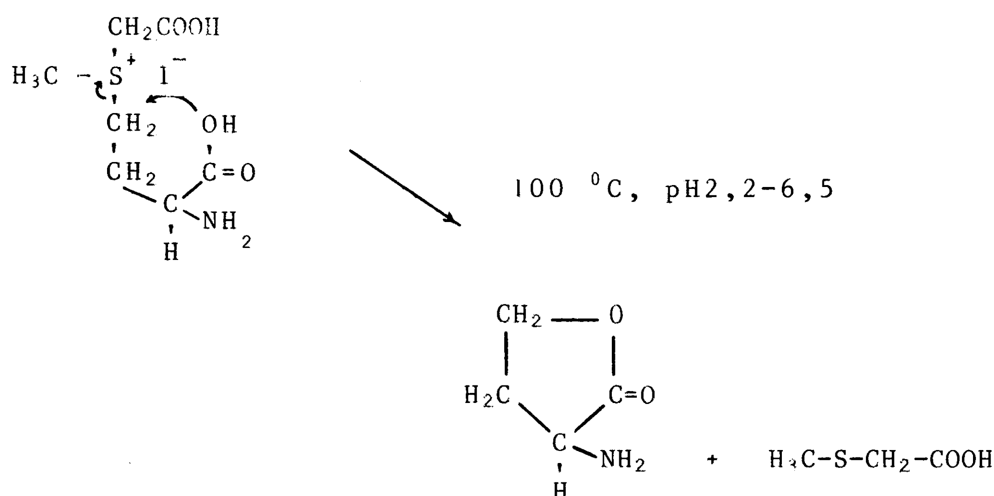
that no S-carboxymethylcysteine had formed indicating that no free sulf= hydryl groups were present. Molecular mass determination by sedimentation equilibrium centrifugal analysis revealed that dissociation had taken place during the above treatment. The results are shown in Table 10.

2.4.8 Cyanogen bromide cleavage

Methionine sulfonium salts may decom= pose in a number of ways, depending on the reaction conditions and the nature of the S-alkyl groups. Of par= ticular interest in this respect is the decomposition of methionine carboxy= methylsulfonium iodide which may arise during S-carboxymethylation of cysteine residues of proteins (114) (Scheme 6).



Scheme 6. Decomposition of methionine carboxymethylsulfonium iodide.

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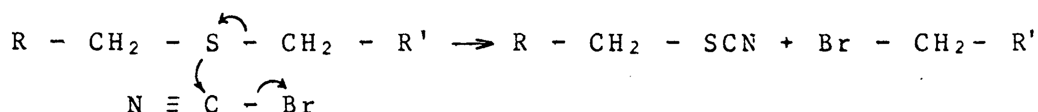
The decomposition of the sulfonium salt is clearly an intramolecular process in which the carboxyl group participates. Since the carbonyl group of a methionine peptide may also displace the sulfur function as methyl carboxymethylthio-ether it is evident that the decomposition is useful for inducing selective peptide cleavage. However, when iodoacetic acid is the alkylating agent, elevated temperatures and low pH values are re= quired. To make the cleavage specific at methionine peptide bonds the sulfonium salt forming reagent should react speci= fically with methionine under appropriate conditions. Furthermore, to make the method applicable to the cleavage of proteins, the methionine sulfonium in= termediate should be sufficiently labile to undergo intramolecular lactonization under mild conditions of pH and tempera= ture.

A reagent which satisfies these require= ments is cyanogen bromide (115). This reagent was used by von Braun and Engel= bertz (116) for the cleavage of thio-ethers (Scheme 7).



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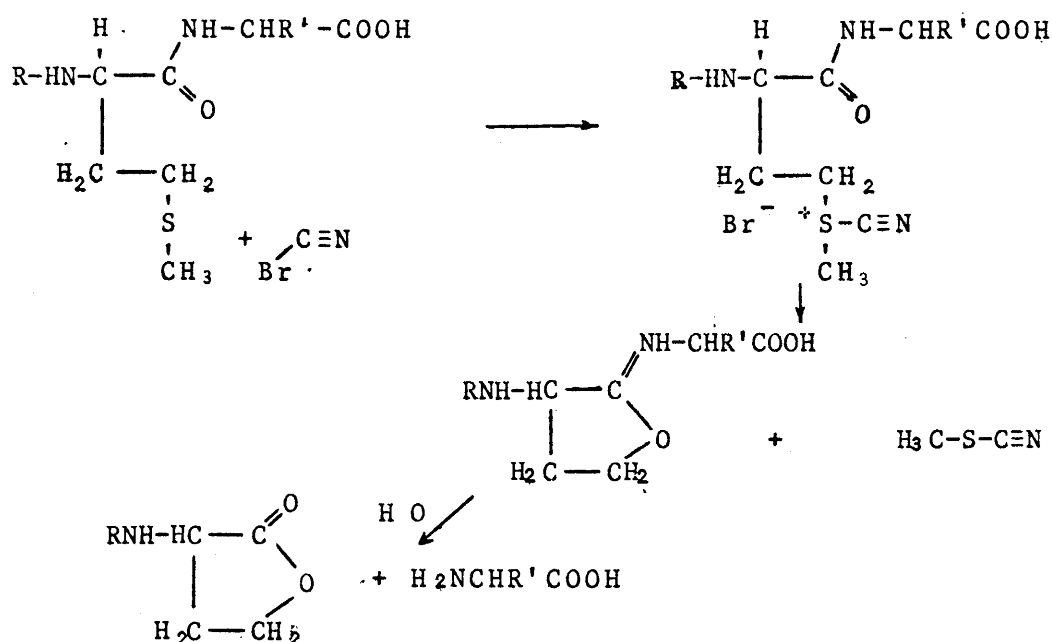
Scheme 7. Cleavage of thio-ethers by cy= anogen bromide.

The reaction requires elevated temperatures for completion. By contrast, cyanogen bromide reacts with methionine at room temperature because the carboxyl group exerts a strong neighbouring group effect (Scheme 8)



Scheme 8. Cleavage of methionine peptide bonds by cyanogen bromide.

At least three seperate reactions are in= volved :

(i) formation of a sulfonium compound

(ii) cyclization to an imidate and

(iii) hydrolytic cleavage of the imidate. The cyanosulfonium bromide of methionine has not been isolated. The imidate formed in the reaction of CNBr with N-acetyl-D,L-methionylglycine has been isolated and identified by Inglis and Edman (118), by

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performing the reaction under anhydrous conditions. By using anhydrous conditions and gaschromatographic monitoring of the methylthiocyanate released during imidate formation, the effects of neighbouring groups on the cyanogen bromide reaction was studied by these authors. They showed that CNBr showed decreasing reactivity with the following compounds : N-acetyl=methionylglycine> N-acetylmethionine methionine> methionylalanine. It, therefore, appears that a free amino group on the methionine residue has a inhibitory effect on the cyclization reaction. This inhibitory effect could arise when a methio=nine residue is in a N-terminal position or when 2 methionine residues are adjacent to each other. The more rapid reaction of methionine in comparison to methionyl=alanine could be the result of the strong neighbouring group effect excerted by a free carboxyl group, which facilitates the reaction.

It should be borne in mind that quantita=tive yields of methylthiocyanate do not necessarily indicate that the overall reaction of CNBr with methionine peptides (Scheme 8) has gone to completion since the imidate may not always be readily hydrolyzed.

The selectivity of the reaction of CNBr with amino acid residues is pH depen=

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dent. Reactions with the primary amino groups of amino acids start in the pH range 5,8 to 6,5 (119). At higher pH values, the reaction is enhanced. However, at pH values progressively higher than 7, CNBr is decomposed. Under suit= able acidic conditions only methionine and cysteine of the commonly occurring amino acids react. Cysteine is slowly oxidized to cysteic acid. Since S-car= boxymethylcysteine does not react with CNBr, cysteine may be rendered unreactive by carboxymethylation. Sulfhydryl groups may also be protected reversibly by benzylation. S-benzylcysteine does not react with CNBr. Not all S-alkyl deriva= tives of cysteine are unreactive towards CNBr. For example S-methylcysteine con= taining peptides may be cleaved with CNBr at elevated temperatures by a β-elimina= tion reaction to form a dehydroalanyl peptide which is subsequently cleaved to form a pyruvyl peptide. At low tem= peratures an oxazoline ring is formed. This is also the intermediate postu= lated for the N→O-acyl shift of serine residues in peptides (120). In acid the oxazoline ring is opened to form an O-acyl derivative of serine. Acid hydrolysis of the ester bond leads to the formation of a serine residue

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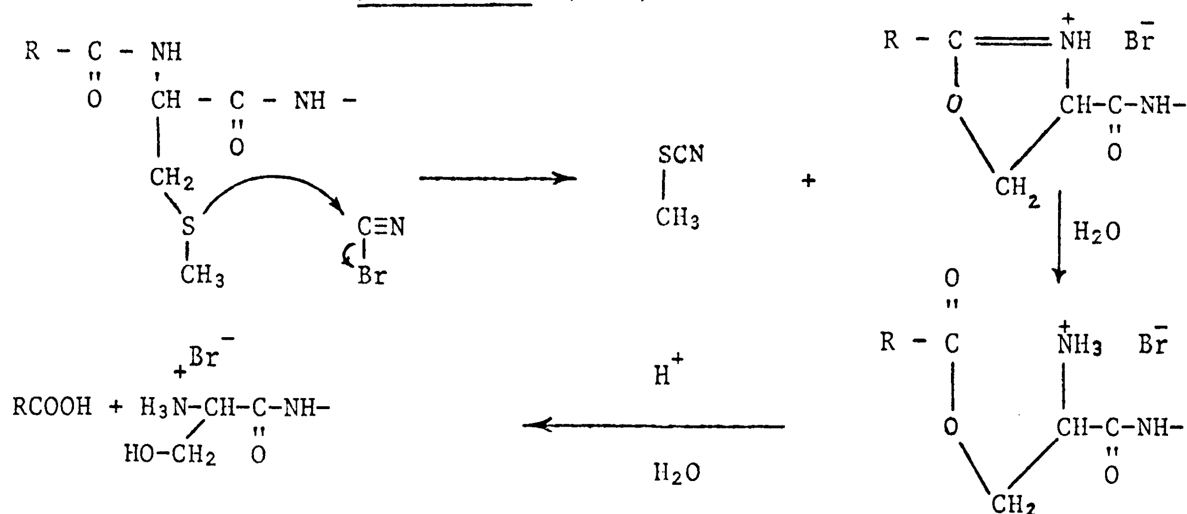
(Scheme 9) (121).



Scheme 9. Cleavage of S-methylcysteine bonds by cyanogen bromide.

Additional sites for cleavage by CNBr may thus be produced by preparation of S-methylcysteine residues. This may be accomplished by either S-methylation of cysteine residues with methyliodide (122) or by converting dehydroalanine residues with methylmercaptan to S-methylcysteine residues. In this investigation the re= action conditions described by Gross & Witkop (123) were employed for the cleavage of the CM-toxic and CM-non-toxic components. The reaction was performed with between 0,45 and 1,45 μmol of CM-protein and a 30-fold ex= cess of CNBr on a molar basis.

The solvent was either 0,1N HCl or 70% HCOOH. The required quantity of CNBr was weighed in a glass-stoppered tube (capacity 1 or 3 ml) and the protein solution (1 μmol of protein / 2 ml solvent) added. After

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the CNBr had dissolved, the reaction mixture was left at room temperature for 24 hours. Initially, O,1N HCl was used as solvent. The CM-toxic and CM-non-toxic proteins, however, formed some insoluble material during the reaction. In addition, the proteins dissolved very slowly. Formic acid (70%) proved to be a much superior sol= vent, since the CM-proteins dissolved completely in a short time and no in= soluble material formed during the reaction. After the reaction period, the excess CNBr, methylthiocyanate and solvent were removed by lyophilization. The recovery of the proteins were in all cases essentially quantitative. The extent of the cleavage reaction was de= termined by amino acid analysis on 0,8 - 1 mg samples (Table 13). The analyses were performed as described previously (See Section 2.4.2).

The amino acid analyses showed a decrease in methionine and the appearance of peaks at the homoserine and homoserine lactone positions. The decrease in methionine was used to calculate the extent of the cleavage reaction.

The usual time for the reaction of CNBr with various proteins reported in the

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TABLE 13 EXTENT OF CLEAVAGE OF VARIOUS SAMPLES BY CYANOGEN BROMIDE

Sample	Solvent	Extent of Cleavage in %
CM-Toxic component	70% HCOOH	77
CM-Non-Toxic compo=nent	70% HCOOH	78
CM-RNase	0,1N HCl	80
Standard amino acid mixture	0,1N HCl	99

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literature is 24 hours at room tempera= ture. Inglis & Edman (118) studied the reaction of CNBr on glucagon and strep= tokinase. They found an almost com= plete recovery of methylthiocyanate with= in 0,75 hours in both cases. It thus seems that the reaction time can be dras= tically reduced. It was thus decided to investigate the possiblity of reduc= ing the time under the reaction conditions employed in this investigation. CM- Ribonuclease was chosen as model protein. Production of MeSCN which was determined gaschromatographically was used as criterion of the extent of the reaction. The re= action between CNBr and CM-RNase was per= formed as described above. At suitable time intervals samples were withdrawn with a microsyringe and methylthiocyanate determined gaschromatographically. A Perkin Elmer Model 880 gaschromatograph with a coiled glass column (183x0,4 cm (inside diameter)) packed with 4% carbowax 1000 on chromosorb W was used. The temperature of the column was 95 ^{0}C, that of the injection port 145 ^{0}C. Ni= trogen was used as carrier gas. The flow rate was 27 ml min^{-1}.

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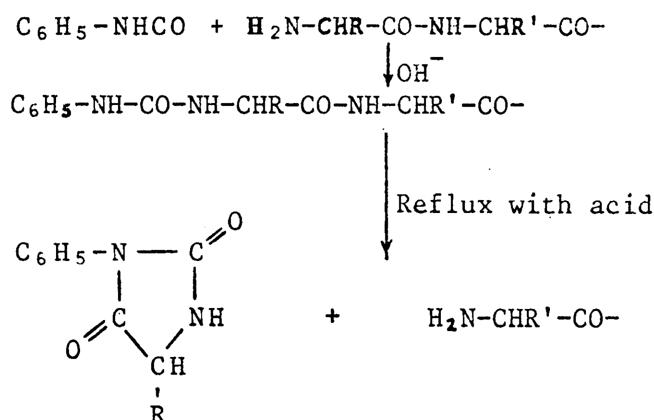
The methylthiocyanate produced during the reaction of CNBr with RNase is shown in Fig 16.

It is evident that if methylthiocyanate production is positively correlated with CNBr cleavage of RNase, the time for the reaction may be decreased considerably.

Several attempts were made to separate the peptides formed as a result of the cyanogen bromide cleavage. However, the results obtained cannot be consoli= dated at this stage without further re= search and will not be reported.

2.4.9 Amino acid sequence determination

The method devised by Edman for the amino acid sequence determination of proteins and peptides is based upon the reaction of amino acids with phenylisocyanate (Scheme 10). This reaction was described in 1927 by Bergmann & Miekeley (124).



Scheme 10. Reaction of phenylisocyanate with peptides and proteins.

An important property of the reactions,

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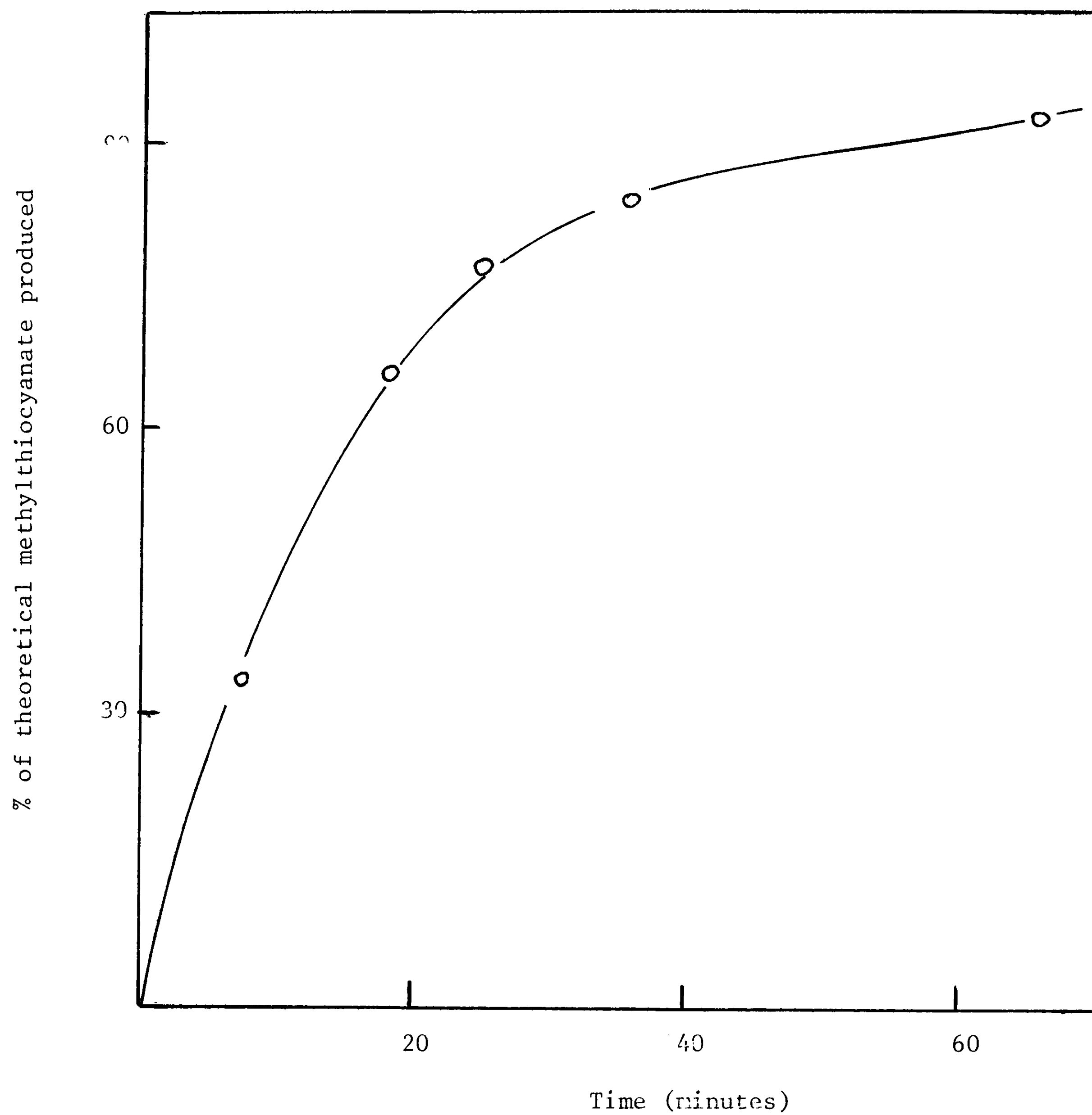

Fig 16 Methylthiocyanate produced during the reaction of cyanogen bromide with ribonuclease.

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shown in Scheme 10 is that the phenyl=
carbamyl group poses a labilizing effect
on the peptide linkage of the N-terminal
amino acid. This is indicated by the
fact that this bond is more readily
hydrolyzed by acid in comparison to most
normal peptide bonds. This obviously im=
plicates the usefulness of the reaction
for amino acid sequence studies of pep=
tides and proteins. Indeed, Abderhalden
& Brockmann employed this method for the
stepwise degradation of polypeptides in
1930 (125). However, they found that,
apart from the expected cleavage of the
N-terminal peptide bond, hydrolyses of
other peptide bonds occurred. This led
Edman to introduce phenylisothiocyanate
instead of phenylisocyanate in the above
reactions (126, 136). Edman reasoned
that the phenylthiocarbamyl peptide would
more readily form the phenylthiohydantoin
derivative of the N-terminal amino acid
in comparison to the corresponding phenyl=
hydantoin formation from the phenylcarbamyl
peptide. This implicates less drastic
conditions for the cleavage of the N-
terminal peptide bond which reduces the
danger of other peptide bonds being
cleaved. Furthermore, Edman proposed
the use of an anhydrous medium for the

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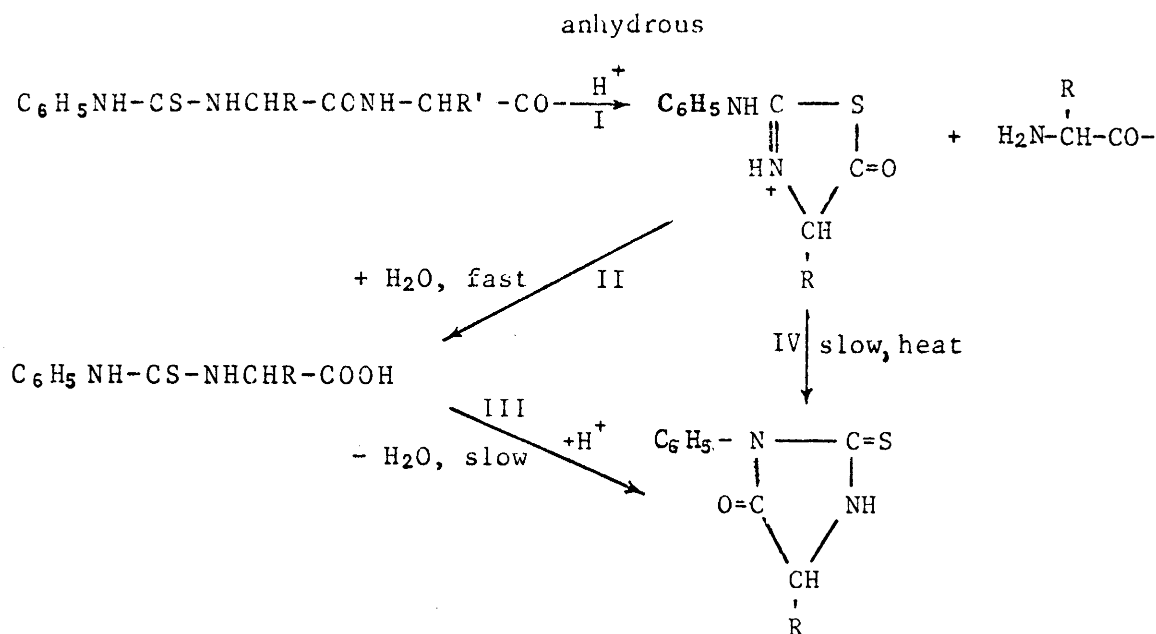

Scheme 11. Reactions during the conver= sion of PTC-peptides to N- terminal PTH-amino acids.

Edman showed that reactions I and II are fast reactions, whereas reactions III and IV are comparatively slow. Thus the exposure of the peptide to the anhydrous acid (reaction I) may be li= mited to the time necessary for the completion of this reaction. The formed thiazolinone may then be separated from the peptide and converted by aqueous acid to the isomeric PTH-derivative, thus obviating the need of exposure of the peptide to possible hydrolytic action by the acid in aqueous medium (135).

The degradation mechanism thus involves three separate reactions :

1 Coupling. The N-terminal amino acid reacts with PITC to form the PTC-pep= tide.

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2 Cleavage. The PTC-peptide is cleaved to form the 2-anilino-thiazolinone derivative of the N-terminal amino acid and the peptide with the N-terminal amino acid removed.

3 Conversion. The 2-anilino-thiazolinone amino acid is converted to the PTH-amino acid.

These fundamental investigations into the mechanism of the above reactions (126, 129, 131, 135) eventually enabled Edman to establish the sequence of up to 60 amino acids (138).

The distinct features of the three-step degradation procedure and problems asso= ciated with each step were outlined by Edman (138, 139) and by Niall (140, 141). Some of these are presented below:

1 Coupling :

The pH of the reaction medium should be sufficiently high since only the unprotonated amino groups of the amino acids react. At pH below 8,5 the α-amino groups become largely protonated and at a pH above 10 destruction of the PITC occurs. A buffer is required since hydrogen ions are produced in the coup= ling reaction. The buffer should (a) have a pk_a close to 9, (b) be volatile and (c) not react with the PITC.

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Furthermore, the buffer medium should serve as solvent for both the non-polar PITC and the protein or peptide. Suit= able buffers have been described (138, 139, 140).

Several side reactions may occur during the coupling. An important one, which leads to N-terminal blocking, is caused by desulfuration of the phenyl= thiocarbamyl to the phenylcarbamyl protein which does not form the phenyl= hydantoin derivative under the usual conditions of the Edman degradation (142). This oxidation may be due to oxygen dissolved in the reaction medium or oxidants present as im= purities.

The α-amino groups may react with several blocking reagents. Aldehydes present as impurities, for example, may lead to Schiff base formation. Some blocking reactions may be reversible (140). Thus a blocked α-amino group during the coupling stage may be con= verted to the unblocked group during the next coupling. This causes over= lap of consecutive steps.

In addition to reactions of phenyl= isothiocyante with primary amino groups and secondary amino groups of the pro=

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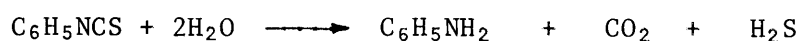
lines, reactions with the imidazole group of histidine, the phenolic group of tyrosine and the free sulfhydryl group of cysteine are possible (143). The latter reaction subsequently leads to cleavage of the peptide chain at cysteine residues. Suitable blocking of the sulf= hydryl groups is thus essential prior to the coupling reaction.

Hydrolysis of the PITC may occur as a side reaction.



$$C_6H_5NCS + 2H_2O \longrightarrow C_6H_5NH_2 + CO_2 + H_2S$$

$$C_6H_5NH_2 + C_6H_5NCS \longrightarrow C_6H_5NHCSNHC_6H_5$$

The S-diphenylthiourea may interfere with the identification of the PTH-amino acids. Byproducts formed during the coupling reaction may be removed by extraction with organic solvents. Considerable loss of shorter peptides, especially those which consist mainly of hydrophobic amino acids, may occur during the extraction.

2 <u>Cleavage</u> :

Two prominent features of the cleavage re= action are that it is rapid and non-hydro= lytic. Some side reactions are possible. When a glutamine residue appears at the N-terminal position after the cleavage re= action, cyclization may occur, forming a pyroglutamic acid residue (144, 145).

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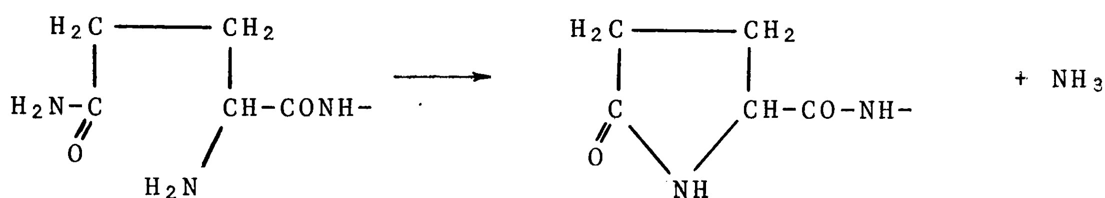
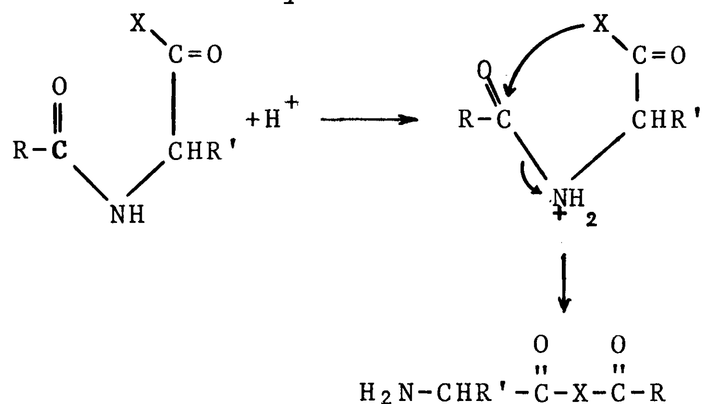


Side reactions involving serine and threonine residues (N→O acyl migration) (146) and aspartic acid residues (α→β peptide shifts) (144) may occur. Furthermore, incomplete and non-specific cleavage may be encountered. The incomplete cleavage is probably not due to insufficient reaction time but to an equilibrium being established before the occurrence of complete reaction (138). The non-specific cleavage may be accounted for by acidolysis of peptide bonds. It has been shown that anhydrous trifluoroacetic acid may cause acidolysis of peptide bonds (147) (Scheme 12). Other anhydrous acids commonly employed in the cleavage reaction may behave in the same way.



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Scheme 12. Acidolysis of peptide bonds under anhydrous conditions showing the participation of carboxyl or N-substituted carboxamide groups (X:OH or NHR").

Prior to the conversion reaction, de= scribed below, the thiazolinone should preferably be separated from the shortened peptide by extraction with an organic solvent. Loss of shorter pep= tides may occur during this process as the result of small differences in solubilities of the thiazolinones and the peptides.

3 Conversion.

The conversion of the thiazolinone to the PTH-amino acid comprises two reactions : hydrolysis of the thia= zolinone to the PTC-amino acid fol= lowed by cyclization to the PTH-amino acid under acidic conditions (Scheme 11).

Kinetic studies of the latter reaction have revealed that the rate increase, observed with increasing hydrogen ion concentration, varies depending on the amino acid (142). Optimal conditions with regard to hydrogen ion

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concentration, temperature and time for routine conversion of the thiazoli= nones of all the amino acids have been described (142). High yields of the PTH-amino acids may be obtained with a few exceptions. The serine and to a lesser extent the threonine PTH-deri= vatives undergo β-elimination. The tryptophan derivative decomposes, conver= sion of the glycine derivative is incom= plete and the asparagine and glutamine derivatives are partially deaminated. By rigidly controlling the reaction conditions and thereby eliminating the above-mentioned side reactions exten= sive degradations are possible. Edman & Begg (138) have described an instru= ment which automatically performs the coupling and cleavage reactions. The principle of their automated procedure is that reactants and extracting sol= vents are spread out in thin films inside a rotating cup in the absence of oxygen. The large surface area of the films facilitate the extraction steps and evaporation of solvents or reac= tants.

The identification and quantitation of the PTH-amino acids may be accomplished directly by paper chromatography (130, 148), thin-layer chromatography (138, 149),

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gas-liquid chromatography (150, 151, 154) or mass spectroscopy (152, 153). Alter= natively, the PTH-amino acids may be hydrolyzed by acid or alkali and the formed amino acids analyzed by standard procedures (155, 156). Electrophoretic procedures may be employed to identify PTH-arginine and PTH-histidine (157). Since none of the identification methods is adequate by itself, it is generally necessary to employ a combination of these methods. However, at present gas-liquid chromatography seems to be the best single procedure available for the identification and quantitation of most of the amino acids. In this investigation the amino acids se= quence of the CM-toxic and CM-non-toxic components were investigated with a Beck= man, Model 890, sequencer (Beckman Instru= ments, Palo Alto. California). The in= strument was operated according to the pre= liminary instruction manual (September 1970 Edition). All reagents used in the instru= ment were obtained from Beckman Instruments. A stroboscope was used to enable better observations of the processes occurring in the reaction cup. The sequencer pro= gram used in these investigations is shown in Table 14.

All notations in the program are

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TABLE 14 PROGRAM EMPLOYED FOR THE DETERMINATION OF THE AMINO SEQUENCE OF THE CM-TOXIC AND CM-NON-TOXIC COMPONENT

Beckman®

SEQUENCER

PROGRAM DOUBLE CLEAVAGE

DATE

NAME

TEMP. 50 SETTING

REAGENTS

	TYPE	PRESS.
N_2 Cell		40
R_1	PITC-Heptane	
R_2	Quadrol*	
R_3	HFBA	
R_4		
R_5		
S_1	Benzene	
S_2	Ethyl acetate	
S_3	Butyl chloride	

* Registered Trade Mark Wyandotte Chem. Corp.

Column headings: PROGRAM CONTROL (START SLEW, STOP SLEW, PROGRAM); DRIVE SPEED (HIGH); STEP TIME IN SECONDS (HUNDREDS 800, 400, 200, 100; TENS 80, 40, 20, 10; UNITS 8, 4, 2, 1); REAGENT TRANSFER (REAGENT 1–5); SOLVENT TRANSFER (SOLVENT 1–3); REACTION CELL (PRESSURIZE, RESTRICTED VAC, ROUGH VACUUM, FINE VACUUM, NITROGEN); FRACTION COLLECTOR (VACUUM, STEP); EFFLUENT VALVE (WASTE, COLLECT); PRESSURIZE (R & S BOTTLES); PROGRAM STAGE (STAGE 4, 2, 1); columns 1–42.

PROGRAM STEP	PROGRAM STATEMENTS	STEP TIME	SPEED	PROGRAM STEP
1	Slew Stop	2	Low	1
2	Delay	4		2
3	R_1 Vent	14		3
4	R_1 Press	14	High	4
5	R_1 Deliver	4		5
6	Vacuum -restricted	40		6
7	Delay	4		7
8	Nitrogen dry	100		8
9	R_2 Vent	14		9
10	R_2 Press	30		10
11	R_2 Deliver	55		11
12	Reaction	120		12
13	Reaction	840	Low	13
14	Reaction	840		14
15	S_2 Vent	30		15
16	S_2 Press	30		16
17	S_2 Deliver	4		17
18	Vacuum-restricted	300		18
19	Vacuum-rough	300		19
20	Vacuum-fine	300		20
21	Delay	4		21
22	S_1 Vent	30		22
23	S_1 Press	30		23
24	S_1 Deliver	300		24
25	Nitrogen dry	200		25

Table 14 continued on page 132

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TABLE 14 continued from page 131

Beckman® SEQUENCER

PROGRAM ______
DATE ______
NAME ______
TEMP. ____ SETTING ______

REAGENTS	TYPE	PRESS.
N_2 Cell		
R_1		
R_2		
R_3		
R_4		
R_5		
S_1		
S_2		
S_3		

Column groups: PROGRAM CONTROL (START SLEW, STOP SLEW, PROGRAM); DRIVE SPEED (HIGH); STEP TIME IN SECONDS (HUNDREDS 800, 400, 200, 100; TENS 80, 40, 20, 10; UNITS 8, 4, 2, 1); REAGENT TRANSFER (REAGENT 1–5); SOLVENT TRANSFER (SOLVENT 1–3, PRESSURIZE); REACTION CELL (RESTRICTED VAC, ROUGH VACUUM, FINE VACUUM, NITROGEN, VACUUM); FRACTION COLLECTOR (STEP); EFFLUENT VALVE (WASTE, COLLECT); PRESSURIZE (R & S BOTTLES); PROGRAM STAGE (STAGE 4, 2, 1; 1, 2, 3, 4)

PROGRAM STEP	PROGRAM STATEMENTS	STEP TIME	SPEED	PROGRAM STEP
26	S_2 Vent + Vacuum-restricted	30		26
27	S_2 Press + Vacuum-restricted	180		27
28	Vacuum - rough	30		28
29	Delay	4	High	29
30	Delay	4		30
31	Delay	4		31
32	S_2 Deliver	700		32
33	Delay	60		33
34	Vacuum-restricted	60		34
35	Vacuum - rough	40		35
36	Vacuum - fine, Fraction collector advance	360		36
37	Delay	4		37
38	R_3 Vent, Fraction collector vent	20		38
39	R_3 Press	14		39
40	R_3 Deliver	20		40
41	Reaction	180		41
42	Vacuum-restricted	60	Low	42
43	Vacuum - rough	40		43
44	Vacuum - fine	540		44
45	Delay	4		45
46	S_3 Vent	30	High	46
47	S_3 Press	30		47
48	S_3 Deliver - collect	220		48
49	Delay	40		49
50	Vacuum-restricted	60		50

Table 14 continued on page 133

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TABLE 14 continued from page 132

Beckman®

SEQUENCER

PROGRAM ______
DATE ______
NAME ______
TEMP. ______ SETTING ______

REAGENTS

	TYPE	PRESS.
N_2 Cell		
R_1		
R_2		
R_3		
R_4		
R_5		
S_1		
S_2		
S_3		

PROGRAM STEP	PROGRAM STATEMENTS	STEP TIME	SPEED	PROGRAM STEP
51	Vacuum - rough	20	High	51
52	Vacuum - fine	60		52
53	Delay	4		53
54	R_3 Vent	14		54
55	R_3 Press	14		55
56	R_3 Deliver	20		56
57	Reaction	180		57
58	Vacuum-restricted	60	Slow	58
59	Vacuum - rough	40		59
60	Vacuum - fine	140		60
61	Delay	4		61
62	S_3 Vent	30	High	62
63	S_3 Press	30		63
64	S_3 Deliver - waste	150		64
65	Delay	40		65
66	Vacuum-restricted	60		66
67	Vacuum - rough	20		67
68	Vacuum - fine	600		68
69		2		69
70		2		70
71	Slew start			71
72	Program Conditional Stop			72

PROGRAM CONTROL (START SLEW, STOP SLEW, PROGRAM) · DRIVE SPEED (HIGH) · STEP TIME IN SECONDS (HUNDREDS, TENS, UNITS) · REAGENT TRANSFER (REAGENT 1–5) · SOLVENT TRANSFER (SOLVENT 1–3) · REACTION CELL (PRESSURIZE, RESTRICTED VAC, ROUGH VACUUM, FINE VACUUM) · FRACTION COLLECTOR (NITROGEN, VACUUM, STEP) · EFFLUENT VALVE (WASTE, COLLECT) · PRESSURIZE (R & S BOTTLES) · PROGRAM STAGE (STAGE)

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explained in the sequencer instruction manual.

Several modifications in instrument de= sign, programs and reagents have been reported (158, 159, 160). Some of the modified programs have been investi= gated in this laboratory. However, since model proteins (myoglobin, trypsin and ribonuclease) were used and the investi= gations have not been completed these results will not be reported.

The samples were introduced into the se= quencer reaction cup in the following way : The freeze-dried, salt-free samples (200-250 nmoles) were dissolved in 0,5 ml, 0,2N acetic acid in a small glass vial and delivered by means of a 1 ml syringe with a teflon covered needle into the reaction cup, rotating at low speed (approximately 1000 rpm). During the delivery, the tip of the syringe was held a few millimetres above the bottom and near the wall of the cup to avoid splashing. After the sample had been introduced, the vial was rinsed with 0,1 ml of solvent which was transferred to the cup as described above (the fluid height in the cup was 15mm). The re= action chamber cover was then closed, the scoop placed in the pick-up position and

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the lucite cover placed over the re= action chamber. Hereafter, the reaction chamber heater was turned on. Solvent was removed by evacuation. Restricted vacuum was applied for approximately 10 minutes, followed by a rough vacuum until the sample appeared to be dry. A fine vacuum was then applied to dry the sample completely. This sequence of evacuation was followed to avoid splashing of the sample.

After a pressure of below 7 Pa and a temperature of 50 ^{0}C had been obtained, the program was positioned so as to start at step 60 (See Table 14). The program was started at this particular step, since the butyl chloride precipitated the protein, resulting in a better spreading of the PITC-heptane reagent over the sample in step 5. In addition, the protein film was clearly observable facilitating the setting of the delivery times for the reagents and solvents.

After step 68 the protein had a white dry appearance and was confined to just under the lower half of the cup.

It was found necessary to increase the duration of fine vacuum steps after the cleavage reactions from 140 seconds (the usual time for these steps) to

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540 seconds (Step 44 and 60) since it appeared that the HFBA was inadequately removed with the shorter time. After 540 seconds the pressure was 9 Pa or below. Inadequate removal of the HFBA could result in loss of sample during the butyl chloride extraction. To ascertain if loss of sample material had occurred during the ethyl acetate and benzene extractions, these solvents were collected manually at various stages during the degradation and subjected to amino acid analysis. The results showed that some protein was indeed extracted. The quantities were too low, however, to allow a quantitative evaluation. These investigations became particularly important after an increasing amount of Quadrol buffer was found in the sample tubes and it became necessary to increase the time for the ethyl acetate extrac= tions.

Butyl chloride extracts, containing the thiazolinone derivatives of the amino acids, were dried either with the nitro= gen-vacuum system of the sequencer frac= tion collector or under nitrogen with a nitrogen-evaporator. Conversion of thiazolinone amino acids to the PTH-amino acids was performed according to pro=

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cedures described by Edman and Begg (138): To the dried fractions, 0,2 ml of 1N HCl was added with an Oxford pipettor. Nitro= gen was then blown into the tube for approxi= mately 30 seconds at room temperature and tightly stoppered with a rubber stopper which was covered with acid and alkali resistant parafilm. After mixing with a test tube mixer the conversion was performed in a waterbath at 80 ^{0}C for 10 minutes. The PTH-amino acids were extracted twice from the cooled acid at room temperature with 0,7 ml of peroxide free ethyl acetate. The ethyl acetate was delivered with an aluminium covered Oxford pipettor. (Frequent tests for peroxides with potassium iodide were performed. Freshly opened bottles of ethyl acetate were always tested. Peroxide-free reagents were stored under nitrogen in the dark). Extraction was effected by mixing of the two phases with a test tube mixer for 45 seconds. The phases were separated by brief cen= trifugation. The organic phase was sucked off with a 1 ml Oxford pipettor, and transferred to a 4 ml glass-stoppered tube and evaporated to dryness under nitrogen at 30 ^{0}C.

Identification and quantitation of all

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the PTH-amino acids, excepting arginine and histidine were achieved by gas chroma= tography. Hydrolysis of the PTH-amino acids followed by amino acid analysis was performed to aid in the identification of some of the residues.

Gaschromatographic analysis was performed with a Beckman GC4 gaschromatograph equip= ped with hydrogen flame detectors, double column oven with direct column injection inlets (with teflon septa) and temperature programmer. Initially, prepacked 122 x 0,2 cm (Internal diameter) U-shaped glass columns containing the stationary phases DC-560 and AN-600 were used. The prepacked columns and the stationary phase SP-400 were purchased form Beckman Instruments, Fullerton, California. The SP-400 columns were prepared as follows : U-shaped glass columns (122 x 0,2 cm, internal dia= meter) were washed with concentrated HCl (AR grade), rinsed with distilled water followed by an acetone rinse and dried at 140 ^{0}C. The columns were then silanized by treatment with 5% dichlorodimethylsilane in toluene (150). The columns were filled with this solution and immediately rinsed, first with anhydrous methanol followed by acetone and dried at 140 ^{0}C. After

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the columns had cooled they were filled with stationary phase by means of a small glass funnel. Uniform packing was achieved by gentle tapping of the column with a finger. An electric vibrator was never used since this could damage the packing material. The column was filled between marks made 10 cm from the inlet end and 2 cm from the outlet end. The outlet end was plugged with glass wool. Glass wool plugs were silanized by emer= sion in 5% dichlorodimethylsilane in toluene, followed by washing with anhy= drous methanol and acetone. The plugs were dried at 80 ^{0}C. Initially the inlet end was also plugged. This was omitted, however, since carbonaceous ma= terial accumulated on these plugs. This may lead to absorption of PTH-amino acids or may lead to their decomposition (141). Without the plugs, black deposits appeared on the column material at the inlet end and on the glass walls in this area.after continuous use. These deposits were re= moved by aspiration of the top 1 cm packing material and by gentle cleaning of the glass walls with a pipe cleaner and acetone.

The columns were conditioned in the gas= chromatograph in the following way: the inlet end of the columns were connected

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to the instrument and nitrogen passed through the columns at a flow rate of 120 ml min^{-1}. The oven temperature was set to 50 ^{0}C and after 30 minutes increased to 325 ^{0}C over a period of 320 minutes. This temperature was maintained for at least 16 hours, after which the columns were ready for use.

For the separation of PTH-amino acids the following conditions were used. The flow rates of hydrogen and oxygen for the detector were 45 ml min^{-1} and 300 ml min^{-1}, respectively, and of the nitrogen carrier gas, 120 ml min^{-1}. The inlet tempera= ture was 280 ^{0}C, the detector line tem= perature program was prepared to allow a two minute isothermal period at 165 ^{0}C, followed by a 110 ^{0}C rise over a 16 minute period.

These conditions gave satisfactory results for the identification and quantitation of standard PTH-amino acids which were prepared as follows. The PTH-amino acids (Mann Research or Pierce) were weighed on a Cahn electrobalance and dissolved in 1 ml of ethyl acetate or methanol. The concentration was approximately 5 nmoles ml^{-1} for all the PTH-amino acid standards excepting PTH-glycine and PTH-serine which had concentrations of approximately 10 n moles

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ml^{-1}. TMS-derivatives were prepared by reacting 5 µl of the standard PTH-amino acids with 5 µl N,O-bis(trimethylsilyl) acetamide in stoppered microbench centri= fuge tubes for 3 minutes at 80 ^{0}C. Ali= quots were then injected immediately into the gaschromatograph. Additional peaks were observed for some TMS-derivatives when the time between reaction and injec= tion was prolonged. PTH-amino acids which were dissolved in methanol were evapora= ted to dryness under nitrogen prior to the silylation reaction.

Aliquots of the PTH-amino acids, obtained from the sequencer were injected into the gaschromatograph for identification and quantitation. Aliquots of all the frac= tions were also silylized and injected for the identification of amino acids for which silylation is mandatory and for confirmation purposes. Additional confirmation was obtained in some cases by alkaline hydrolysis and subsequent amino acid analysis of the PTH-amino acids. The hydrolysis was performed in the fol= lowing way : The dried PTH-amino acids were dissolved in 2 ml of 0,1 N NaOH and transferred to a 0,9x15cm hydrolysis tube. Nitrogen gas was bubbled through the solution for 5 minutes and the tube

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connected to an oil vacuum pump by means of a rubber tube in which a three-way stopcock was assembled, enabling the in= troduction of nitrogen into the system. The tube was inserted into an acetone-dry ice mixture. After the tube contents had frozen, the stopcock was positioned to al= low evacuation to a pressure of 13,3 Pa. The stopcock was then postioned to allow the introduction of nitrogen. The procedure was repeated three times after which the tube was sealed under vacuum. Hydrolysis was per= formed at 80 ^{0}C for 12 hours, after which the sample was stored at - 5 ^{0}C. Samples to be analyzed were acidified with 0,2N HCl and dried under nitrogen at 40 ^{0}C. The residues were dissolved in sodium citrate buffer, pH 2,2 and subjected to amino acid analysis (See Section 2.4.2).

In cases where no amino acids could be identi= fied by the gaschromatographic method, the aqueous phase which remained after the ethyl acetate extraction was investigated for the pre= sence of arginine or histidine. To the aqueous phase was added 0,2 ml of 1M Na_2HPO_4, and mixed with a test tube shaker, and extracted twice with 0,7 ml of ethyl acetate as described pre= viously. After evaporation of the organic solvent the residue was dissolved in 50 µl of methanol. An aliquot was injected directly into the gaschromatograph. A second aliquot was silylized before injection and

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and a third was hydrolyzed with alkali and subjected to amino acid analysis as described above. A fourth aliquot was used to perform spot tests for arginine and histidine (Arginine is not detectable by gaschromatographic analysis and is difficult to detect after hydrolysis of the PTH-amino acids). The spot test for arginine was per= formed as follows (161, 141). Aliquots of the sample (5 -10 μl) were spotted with a microsyringe on a strip of Whatman No 3 filter paper and dipped into a mixture containing 5 ml each of 0,2% phenanthrene= quinone in absolute ethanol and 10% NaOH in 60% ethanol. The strips were air dried and observed under an ultravio= let lamp at 266 nm. Arginine containing samples gave a white-blue fluorescence which is characteristic of the quanidino group. The spot test for histidine was performed according to the method of Sanger and Tuppy (162). Aliquots of the sample (5-10 μl) were spotted on Whatman No 3 filter paper. In a hood were mixed 10 ml each of 10% ρ-anisidine in ethanolic O,1N NaOH and 10% iso-amyl= nitrite in ethanol in a spraying reser= voir. The reservoir was connected to a sprayer and after exactly 5 minutes the paper was sprayed. After 10 minutes

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at room temperature the paper was sprayed with 1% KOH in ethanol. His= tidine containing spots developed a rose colour.

The amino acid residues, identified after the CM-toxic and CM-non-toxic components were subjected to amino acid sequence determination described above, are shown in Tables 15 and 16. In Figures 17 and 18, the yields ob= tained at each step are plotted for Cm-toxic and CM-non-toxic components, respectively. In Fig 19 the efficien= cies of the sequence determinations are shown. These were calculated from the alanine residues at residue number 10, 13, 20 and 31 for the toxic and non-toxic components. The efficiency of a standard myoglobin amino acid sequence determination, based on the valine residues at steps 1, 10, 17 & 21 is shown for comparison.

2.5 SOME ENZYME ACTIVITIES IN THE ORAL SALIVARY SECRETION

2.5.1 Proteolytic activity

The proteolytic activity of the crude salivary secretion as well as of various fractions of the secretion were investi= gated with casein and synthetic deriva= tives as substrates.

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TABLE 15 AMINO ACID RESIDUES IDENTIFIED AFTER AUTOMATED EDMAN DEGRADATION OF THE CM-TOXIC AND CM-NON-TOXIC COMPONENTS

CM-Toxic component			CM-Non-Toxic component		
Residue number	Amino Acid	* Yield (%)	Residue number	Amino Acid	* Yield %
1	Gly(Asp)	35(22)	1	Asp	20
2	CM-Cys	45	2	CM-Cys	50
3	Pro	27	3	Pro	30
4	Pro	25	4	Pro	45
5	Gly	48	5	Thr(Pro)	60(7)
6	Val	30	6	Lys	30
7	Pro(Val)	15(6)	7	Pro	20
8	Thr	25	8	Thr(Pro)	40(8)
9	Arg(Pro)(Thr)	- (3)(5)	9	Arg(Thr)(Pro)	-(10)(4)
10	Ala	32	10	Ala	40
11	Tyr(Ala)	30(12)	11	Tyr(Ala)	39(19)
12	Val(Tyr)(Ala)	36(7)(4)	12	Val(Tyr)(Ala)	34(4)(2)
13	Ala(Val)	26(15)	13	Ala(Val)	34(11)
14	Phe(Val)(Ala)	20(7)(15)	14	Phe(Val)(Ala)	42(6)(10)
15	Val(Phe)	20(4)	15	** (Phe)	- (5)
16	Glu(Val)	15(2)	16	Glu	28
17	Gly	24	17	Gly	20
18	**		18	Glu	28
19	Gly	24	19	**	
20	Ala(Gly)	18(11)	20	Ala	23
21	Tyr(Ala)	22(9)	21	Tyr(Ala)	24(9)
22	Leu(Tyr)	15(8)	22	Leu(Tyr)	21(4)
23	Ile	20	23	Ile	21
24	Val(Ile)	18(6)	24	Val(Ile)	21(9)
25	Val(Ile)(Thr)	12(4)(6)	25	Val(Ile)	18(6)
26	Thr(Val)(Ile)	13(8)(3)	26	Ser(Val)	16(8)
27	Leu(Thr)	14(7)	27	** (Ser)	- (5)
28	**		28	Asp	8
29	Leu	11	29	Leu	12
30	**		30	**	

Table 15 continued on page 146

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TABLE 15 CONTINUED

CM-Toxic component			CM-Non-Toxic component		
Residue number	Amino Acid	* Yield (%)	Residue number	Amino Acid	* Yield %
31	Ala	9	31	Ala	13
32	Leu	8			

Overlapping amino acids and their yields are shown in parenthesis.

* Yields calculated from gaschromatographic ana=lysis.

** Multiple peaks were found after gaschromato=graphic analysis at these positions.

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TABLE 16 PARTIAL AMINO ACID SEQUENCE OF THE TOXIC AND CLOSELY RELATED NON-TOXIC COMPONENT DETERMINED BY THE METHOD OF EDMAN (138)

		1 10
Toxic component	*(TC)*	*Gly-CMCys-Pro-Pro-Gly-Val-Pro-Thr-Arg-Ala-*
Non-toxic component	(NTC)	Asp-CMCys-Pro-Pro-Thr-Lys-Pro-Thr-Arg-Ala-
		11 20
	(TC)	*Tyr——Val-Ala-Phe-Val-Glu-Gly- - -Gly-Ala-*
	(NTC)	Tyr——Val-Ala-Phe- - -Glu-Gly-Glu- - -Ala-
		21 30
	(TC)	*Tyr——Leu-Ile-Val-Val-Thr-Leu- - -Leu- - -*
	(NTC)	Tyr——Leu-Ile-Val-Val-Ser- - -Asp-Leu- - -
		31
	(TC)	*Ala——Leu-*
	(NTC)	Ala-

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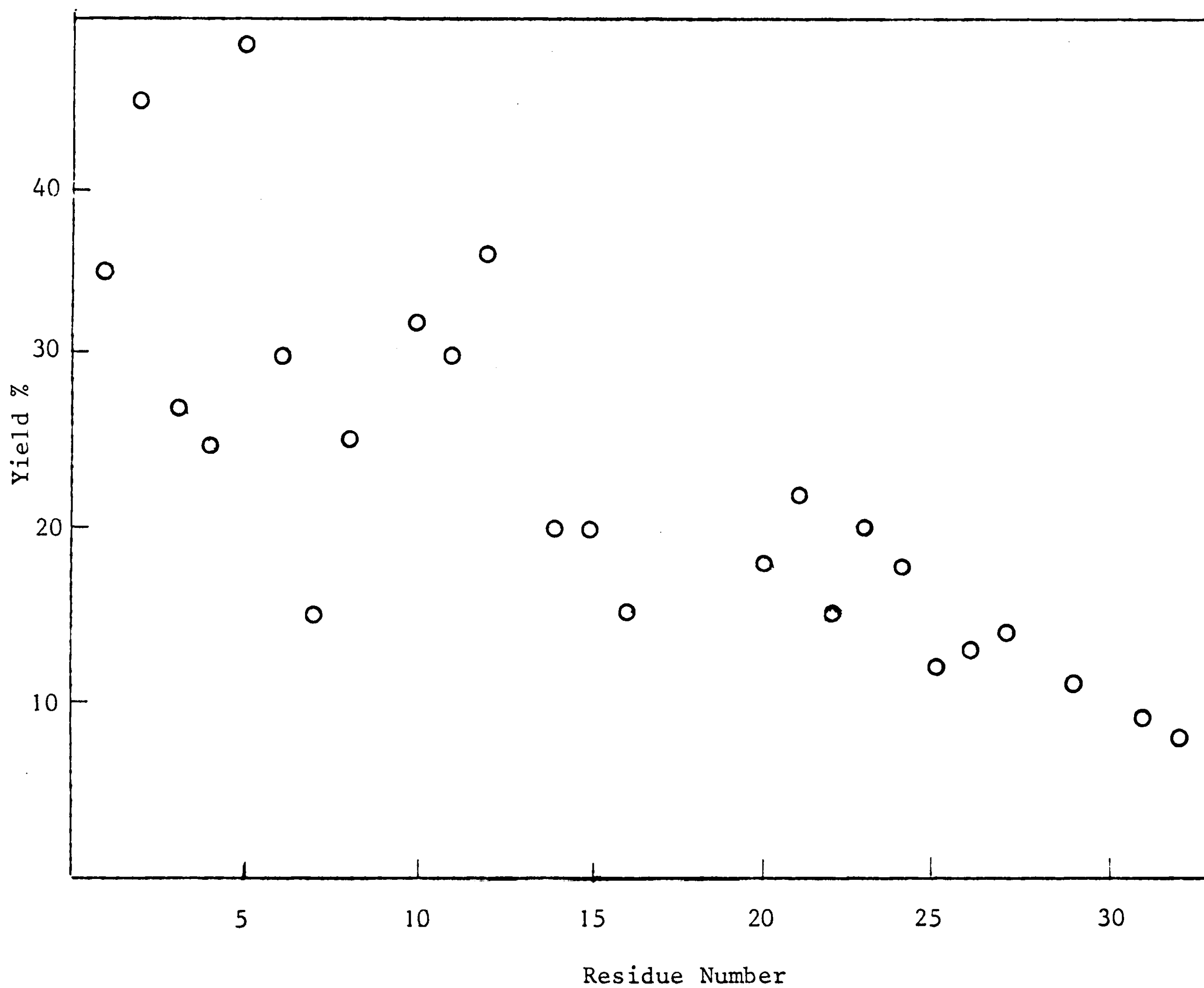

Fig 17 Yields (%) of amino acid residues obtained from the CM-toxic component after each cycle of the automated Edman degradation.

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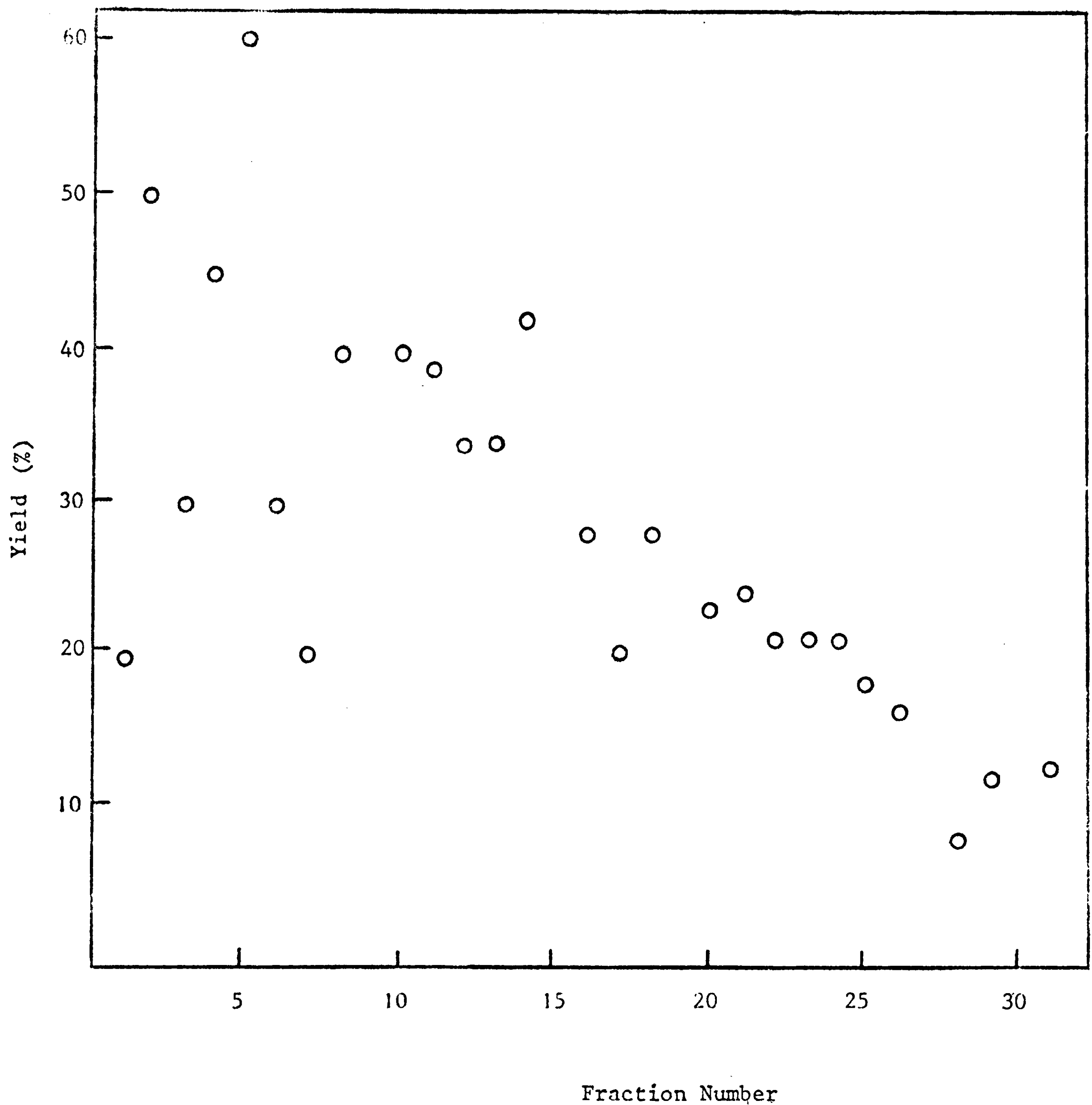

Fig 18 Yields (%) of amino acid residues obtained from the CM-non-toxic component after each cycle of the automated Edman degradation.

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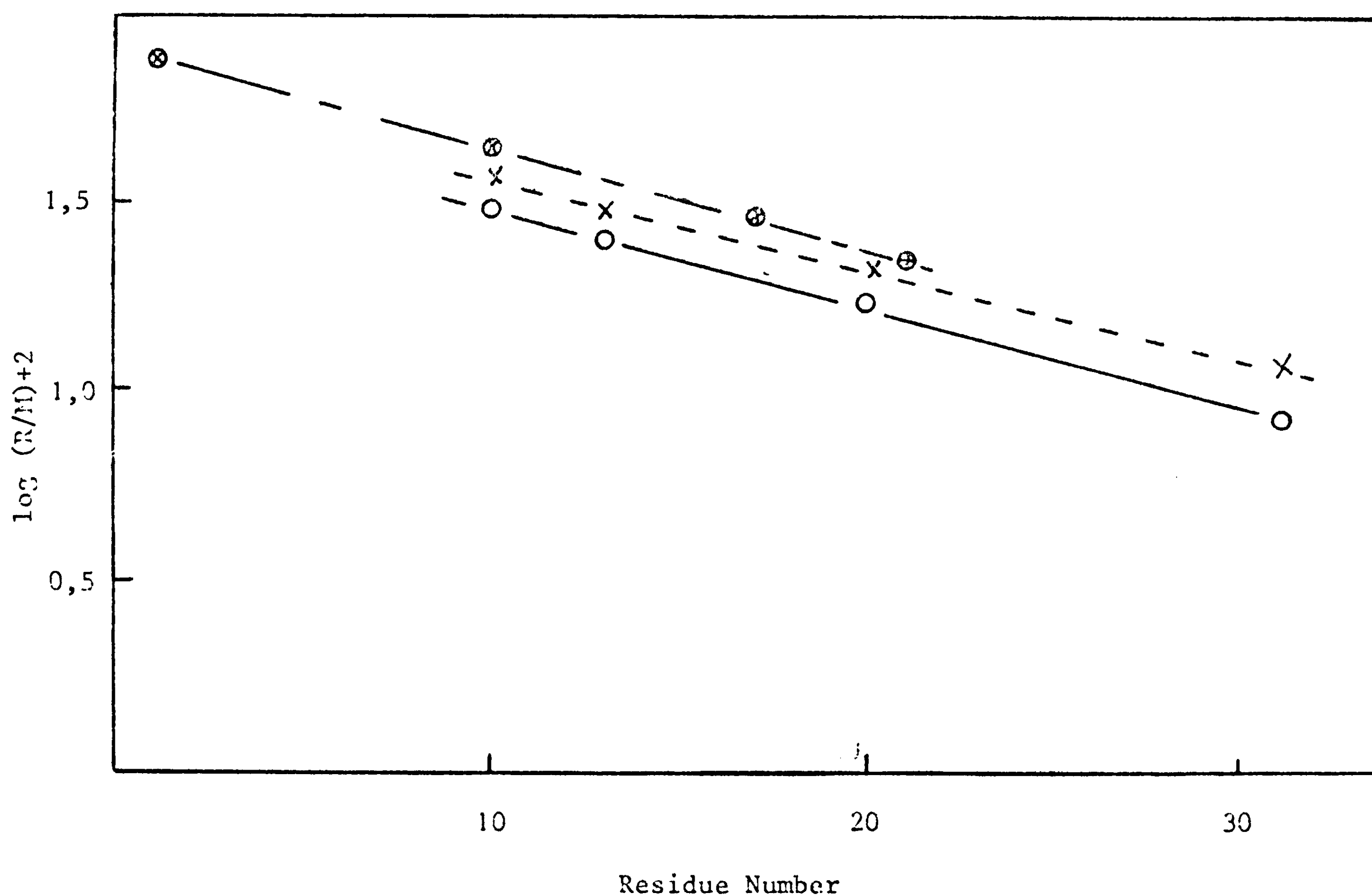

Fig 19 Efficiencies of the amino acid sequence determinations. The log (mol residue per mol protein) or log (R/M) values are plotted against the residue numbers. Alanine residues at cycles 10, 13, 20, and 31 were used; toxic component (— o — o —) and non-toxic component (--X--X--) respectively. For myoglobin, valine residues at cycles 1, 10, 17 and 21 (-- ⊗ — ⊗ -- ⊗) were used. The efficiencies were calculated from the general equation $[(R/M)_x/(R/M)_{x+y}]^{1/y}$, where $(R/M)_x$ is the residue per mol at residue number (x+y). (R/M =(% Yield/100).
The efficiency values were found to be, 87,9; 94,6 and 98,3% for the CM-toxic and CM-non-toxic components and myoglobin, respectively.

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2.5.1.1 Casein as substrate

The protein digestion method of Kunitz (163) as described by Davies and Smith (164) was employed.

Various quantities of the salivary se= cretion were added to a series of test tubes containing 5 ml of 2,5% casein in 0,02M Tris, 0,02M NaCl, pH 7,9 buffer. The tubes were incubated at 37 °C.

At appropriate time intervals test tubes were removed, 10 ml of 0,3M trichloro= acetic acid added and heated in a boiling waterbath for 20 minutes. The mixtures were then filtered through a Whatman No 3 filter paper. A 5 ml aliquot of the clear filtrate was diluted with 10 ml of distilled water and the optical density determined at 280nm using a blank prepared in the following manner. An appropriate quantity of salivary secretion was added to a mix= ture of 10 ml of trichloroacetic acid and 5 ml of the casein solution. The protein was coagulated and removed by filtration as described above.

In Fig 20, a plot is shown of the optical density increase at 280nm with time for two quantities of salivary secretion.

A plot showing the optical density in= crease after 90 minutes digestion of

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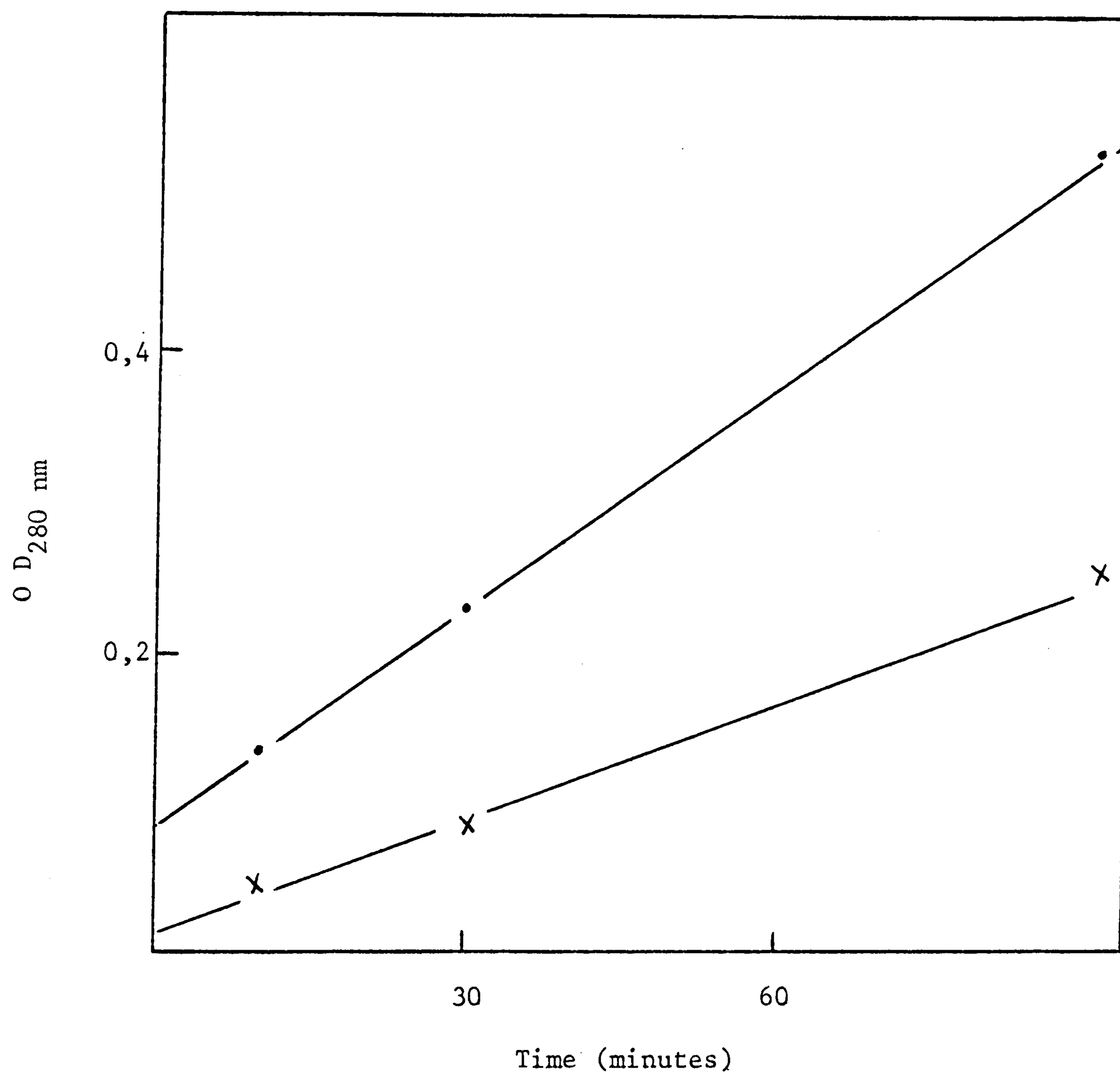

Fig 20 Increase in absorption at 280 nm with time during the digestion of casein with 0,1 ml (x) and 0,2 ml (•) of salivary secretion.

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casein with various quantities of the salivary secretion is shown in Fig 21. A unit of activity was expressed in a manner analogous to the tryptic unit defined by Kunitz (163) viz one unit being the activity which gives rise under the conditions described to an increase of one unit of optical density at 280nm per minute of digestion. The specific activity of the salivary se= cretion (activity per millilitre sali= vary secretion), calculated from the data shown in Fig 21, is $2{,}8 \times 10^{-2}$.

2.5.1.2 <u>N-Benzoyl-L-arginine ethyl ester as substrate</u>

N-Acyl-L-arginine esters may be used as substrate for trypsin (165) and the reaction followed by electrometric titration of the carboxyl groups libera= ted. Schwert and Takenaka (166) have found that the absorption of BAEE is much lower at 250nm than that of ben= zoyl-L-arginine. The hydrolysis of the ester may thus be followed spectrophoto= metrically.

The trypsin-like activity of the sali= vary secretion was investigated in the following way: between 10 and 30 µl of salivary secretion was added to 3 ml of 0,25 mM of a BAEE solution in 0,05M borate buffer, pH 8 and a quantity of

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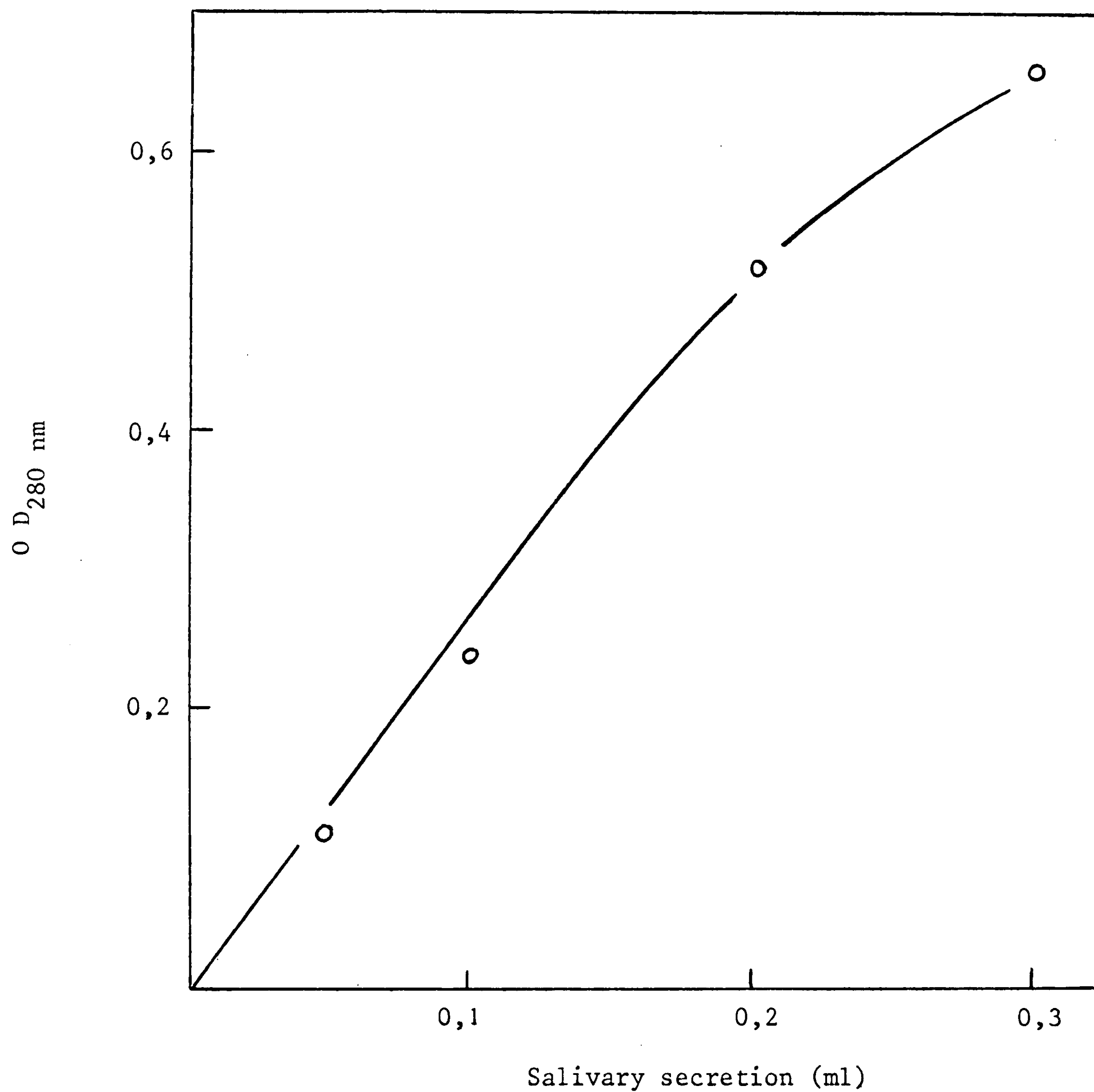

Fig 21 Absorption at 280 nm after a 90 minute digestion period of casein by various quantities of the salivary secretion.

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distilled water to make a final volume of 3,2 ml. A control cuvette contained 3 ml of substrate solution and 0,2 ml of distilled water. The hydrolysis was followed at a wavelength of 253 nm in a Beckman DK2A recording spectrophotometer. The activity, expressed as the µmoles of substrate hydrolyzed per minute per millilitre of salivary secretion was found to be 3,34.

Salivary secretion fractions obtained from the first Sephadex G100 column (Fig 3(c)) were tested using up to 0,24 mg ml^{-1} in the assay mixture. All were devoid of activity. Chromatography of Peak IV obtained from the first Sephadex G100 column (Fig 3 (c)) on a Bio-Rad P10 column (40 x 2,5 cm) with distilled water as eluant, produced 3 peaks (Fig 22) of which Peak I was active. The activi= ty, expressed as µmoles substrate hydro= lyzed per minute per milligram protein was 0,52 and the yield 0,73 mg ml^{-1} salivary secretion.

Since no activity was found in any of the fractions obtained form the Sephadex G100 column (Fig 3(c)) it was reasoned that Peak IV from this column contained an inhibitor which was effectively removed on the Bio-Rad P10 column (Fig 22). This

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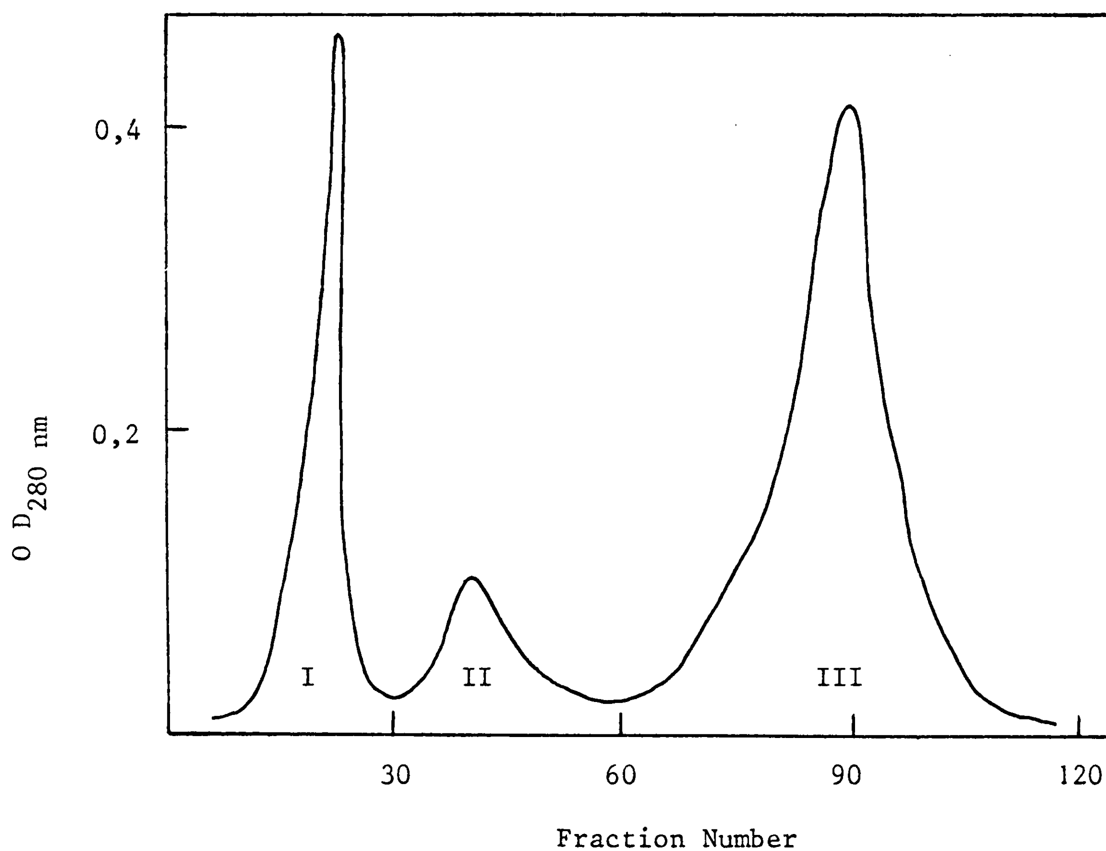

Fig 22 Gel filtration of Peak IV (Fig 3(c)) on a Bio-Rad P10 column (40 x 2,5 cm) with distilled water as eluant. Flow rate : 19 ml h^{-1}, column temperature 8 ^{0}C, and fractions of 2,6 ml collected.

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possible inhibitory effect was tested by adding between 0,063 mg and 2,52 mg of Peak IV (Fig 3(c)) to the assay mixture which contained 115 μg ml^{-1} of Peak I from the Bio-Rad P10 column (Fig 22). Activity measurements where performed as described above. An inhibitory effect was indeed observed as shown in Fig 23. The effect of Peak IV of the Sephadex G100 column (Fig 3(c)) on bovine trypsin (Seravac) was also investigated. The assay was performed as described above with 4 μg of enzyme ml^{-1} assay mixture. No inhibitory effect was observed.

2.5.1.3 Acetyl-L-tyrosine ethyl ester as substrate

The hydrolysis of ATEE by the salivary secretion was followed spectrophotometri= cally at 237nm as described by Tu *et al.* (167).

The substrate (2,95 ml of a 1 mM solu= tion in 0,05M sodium phosphate buffer, pH 7,0) was introduced into a spectro= photometer cuvette and between 50 μl and 200 μl of salivary secretion added. The optical density change was followed at a wavelength of 237nm in a Beckman DK2A recording spectrophotometer. No activity was detected.

Fractions obtained from the first Sephadex G100 column (Fig 3(c)) and from the Bio-

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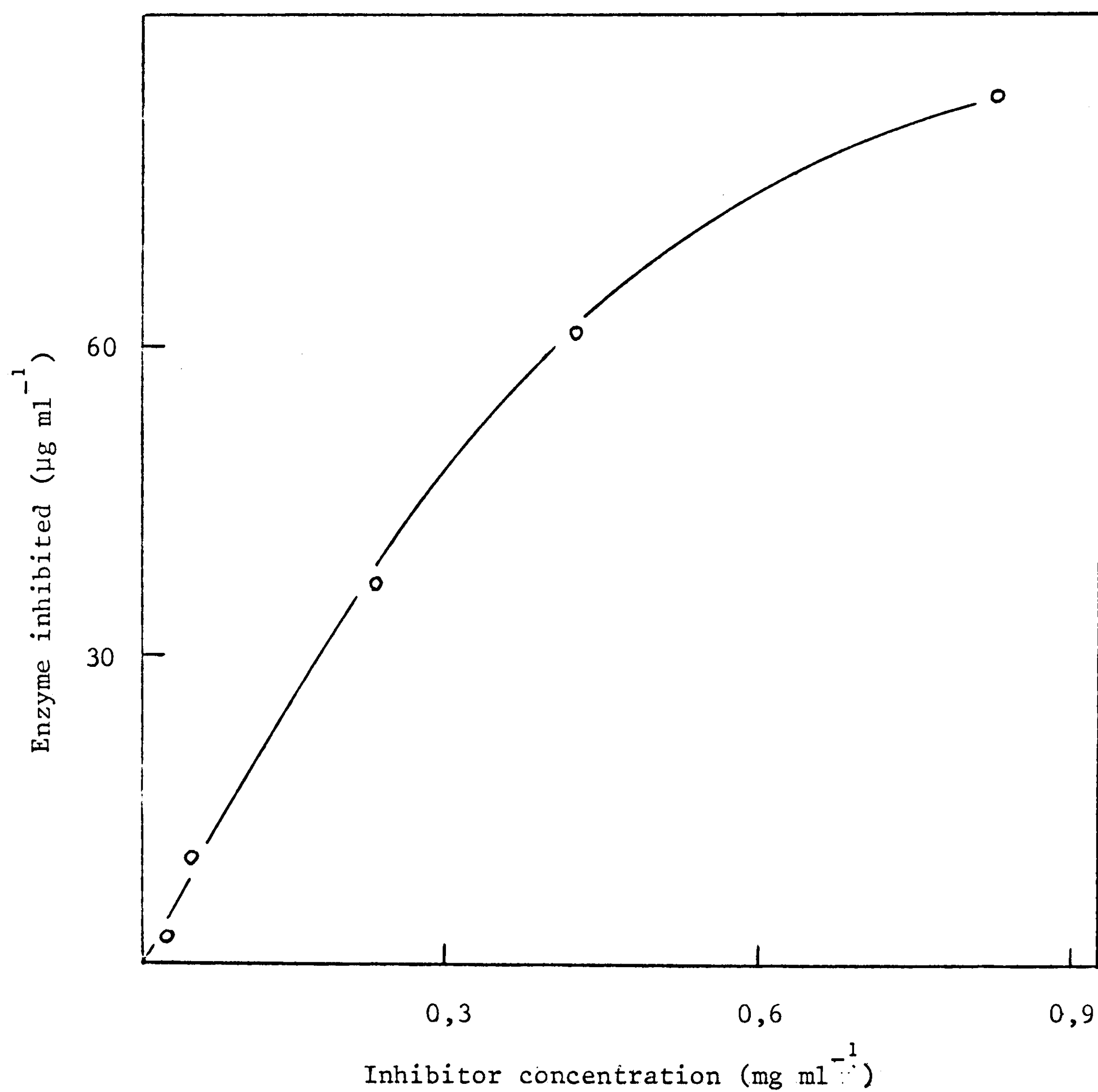

Fig 23 Effect of inhibitor (Peak IV, Fig 3 (c)) on the hydrolysis of BAEE by Peak I, obtained from the Bio-Rad P10 column (Fig 22). Concentration of the enzyme : 115 μg ml^{-1}.

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Rad column (Fig 22) were also assayed for activity. No activity was observed with assay mixtures containing up to 0,5 mg of the fractions.

2.5.1.4 Benzoyl-L-arginine-p-nitroanilide as substrate.

The amidase activity of the salivary secretion was investigated with BAPA as substrate. The method described by Erlanger *et al*. (168) was employed. The BAPA substrate solution (0,9 mM) was prepared as follows. The substrate was dissolved in 0,25 ml of dimethyl=sulfoxide and made to 25 ml with 0,05M Veronal buffer, pH 8. To prevent pre=cipitation of the substrate, the solution was kept at 25 °C. The salivary secretion (50 - 200 µl) was added to 2 ml of the substrate solution and a quantity of Veronal buffer to give a final volume of 3 ml. The liberation of p-nitroaniline was followed spectrophotometrically at 410nm over a period of 5 minutes. The activity of the salivary secretion, ex=pressed as µmol of substrate hydrolyzed per minute by 1 ml of salivary secretion was found to be 0,2.

The activity of Peak I obtained from the Bio-Rad P10 column (Fig 22) was also in=vestigated. No activity was observed

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in assay mixtures containing up to 0,5 mg of this fraction.

2.5.1.5 Acetyl-L-phenylalanyl-L-diiodotyrosine as substrate

Pepsin-like activity of the salivary se= cretion was investigated with APDT as substrate. The assay procedure as described by Ryle (169) was followed. In this procedure the hydrolysis of substrate in 0,01N HCl liberates diiodotyrosine which is estimated by its reaction with ninhydrin. A 1 mM sub= strate solution was prepared as follows. The substrate was dissolved in 1 ml of 0,1N NaOH and made to 10 ml with dis= tilled water. The ninhydrin solution was prepared as described in section 2.2.3.5 (44).

The assay was performed as follows: to 0,5 ml diluted salivary secretion, containing between 0,025 and 0,1 ml secretion in distilled water at 37 °C were added 0,25 ml of 0,05N HCl. After 10 munutes, 0,25 ml substrate solution was added followed by 1 ml of ninhydrin reagent after 20 minutes. The blank solutions were prepared by adding the ninhydrin solution and the substrate solution in reverse order. The tubes were placed in a boiling waterbath for

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exactly 15 minutes and then cooled in a bath of cold water. The contents were then diluted with 5 ml of 60% ethanol and mixed in a test tube mixer. The optical densities of the solutions were determined at 570nm with distilled water as reference. The blank values were subtracted from the test values. The optical density values plotted against the quantity of salivary secretion used in the assay are shown in Fig 24.

The activity, expressed as µmole sub=strate hydrolyzed per minute by 1 milli=lire of salivary secretion was found to be 0,047. The activity of the frac=tions obtained from the first Sephadex G100 column (Fig 3 (c)) and Peak I ob=tained from the Bio-Rad P10 column (Fig 22) were also investigated using up to 0,5 mg of the fractions in the assay mixtures. No activity was found.

2.5.2 <u>Hyaluronidase activity</u>.

Hyaluronidase activity may be determined by biological methods by observing the intradermal spread of coloured indicator solutions (170). Newer methods are based on the polymeric properties of the substrate of the enzyme, hyaluronic acid. These properties are the viscosity of aqueous solutions of the substrate

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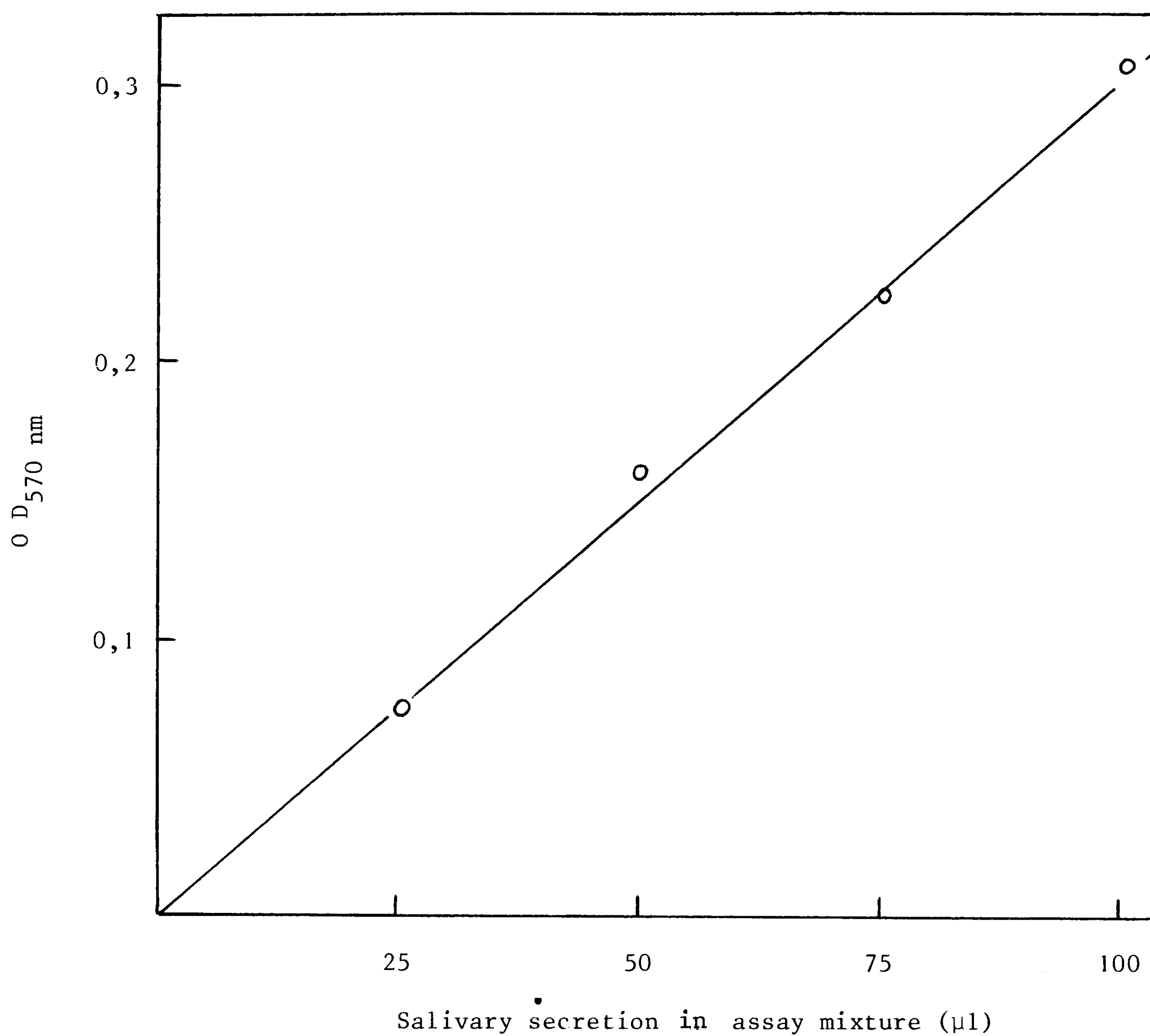

Fig 24 Determination of pepsin-like activity in the salivary secretion with APDT as substrate. Details of the determination are described in the text.

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(171, 172) and the formation of mucin clots with acidified proteins (173). The turbidimetric determination of hyaluronidase activity of Kass and Seastone (174) is a modification of the mucin clot prevention test in which the unreacted hyaluronic acid is determined by the development of turbidity with acidified protein. The latter assay method as described by Dorfman (175) was employed in this investigation. The assay was performed in the following way. A stock solution of umbilical hyaluronic acid (Merck) was prepared by dissolving the substrate in 0,3M KH_2PO_4, Na_2HPO_4 buffer (pH 5,3) so as to obtain a concentration of approximate= ly 4 mg ml^{-1}. A series of dilutions were made which contained from approxi= mately 0,2 to 0,8 mg substrate per milli= litre. To 1 ml of these solutions were added 1 ml of 0,02M sodium phosphate buffer at pH 7 and incubated for 5 minutes at 38 ^{0}C after which turbidity was de= veloped as described below. The trans= mission was determined at 600nm. The dilution which had a transmission of approximately 55% was chosen as the substrate solution for the assays.
A series of dilutions of the salivary

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secretion was made with 0,02M sodium phosphate buffer (pH 7). The solutions contained between 5,6 and 28 µl salivary secretion per millilitre. These solu= tions were mixed with 1 ml of substrate solution and incubated for 45 minutes at 38 °C. After this time period, 10 ml of an acidic ovalbumin solution (0,1% ovalbumin in a solution contain= ing 0,33% sodium acetate and 0,46% glacial acetic acid at pH 3,75) was added rapidly to develop turbidity. After exactly 5 minutes, the optical density at 600nm was determined. The results are shown in Fig 25.

One unit of activity, defined as the quantity of salivary secretion which reduces the optical density by 0,1 O D units under the specified conditions, was found to be 14 µl. The units per millilitre salivary secretion is thus 71,4.

The activity of Peak III, obtained from the first Sephadex G100 column (Fig 3(c)) was also investigated. This Peak show= ed an activity of 5 units mg^{-1}. The yield was 60%.

2.5.3 Acetylcholinesterase activity.

Two colorimetric methods, described by Bergmeyer (176) and Ellman et al. (177)

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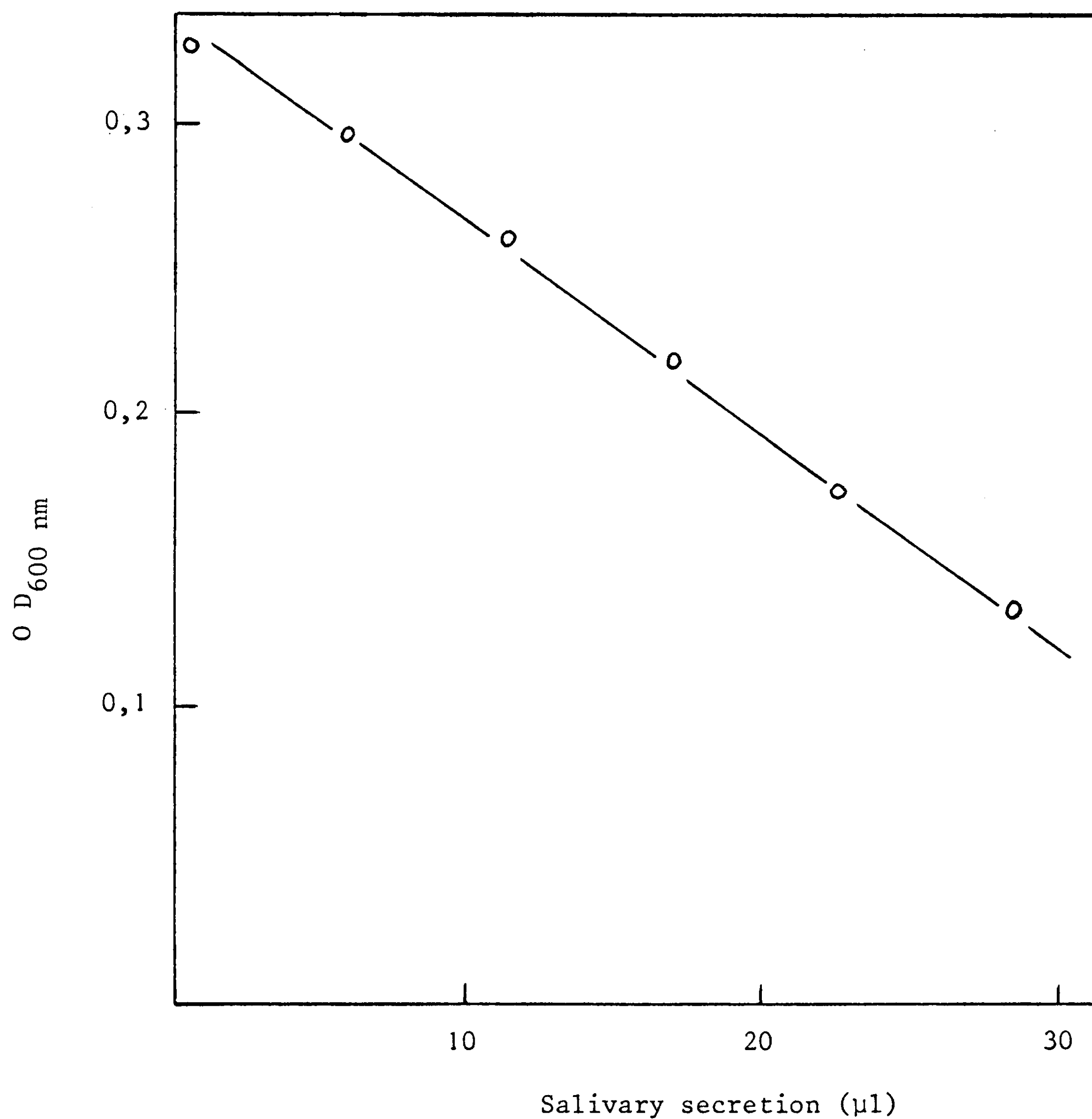

Fig 25 Hyaluronidase activity determination of the salivary secretion by the turbidity method. A decrease in turbidity of hyaluronic acid - protein complexes by the salivary secretion is shown.

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were employed. The activity determination of Bergmeyer is based upon chemical determination of unreacted acetylcholine (178). The ester is reacted with hydroxyl= amine to form acethydroxamic acid which forms a soluble red-purple complex with ferric ions in acid solution. The intensity of the colour is proportional to the concentration of acetylcholine. The substrate solution for the deter= minations was prepared by adding 1 ml of a 0,5M acetylcholine bromide solution and 1 ml of a 0,44M $MgCl_2$, 0,03M KCl solution to 8 ml of 0,1M Veronal buffer at pH 8,2. The acetylcholinesterase activity of the salivary secretion was determined by adding between 100 and 300 µl of secretion to 2 ml of the sub= strate solution. Control tubes con= tained 2 ml of substrate solution and between 100 and 300 µl of distilled water. Reaction mixtures were incubated for 1 hour at 37 °C. After this time period, 2 ml of an alkaline hydroxylamine solu= tion was added. The latter solution was prepared by mixing equal volumes of 14% NaOH solution and a 14% hydroxylamine hydrochloride solution. After 1 minute 6 ml of 0,5N HCl was added and the mixture shaken by hand for a few seconds.

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A 0,05 ml aliquot was withdrawn and in= troduced into a 15 ml capacity centrifuge tube and 10 ml of a 1% $FeCl_3 \cdot 6H_2O$ solution in 0,02N HCl added. After mixing, the tube was centrifuged for 5 minutes at 2000 g and the optical density of the supernatant determined at 540 nm. The amount of acetylcholine was obtained from a standard curve. The standard curve was prepared using 40, 60, 80 and 100 µmoles of acetylcholine bromide. The plot of optical density against µmoles substrate was found to be linear. The activity of the salivary secretion expressed as µmoles substrate hydrolyzed by 1 ml of secretion per minute was found to be 1,85.

The activity determination described by Ellman _et al_. (177) is based upon the measurement of the reate of production of thiocholine liberated during the hydrolysis of acetylthiocholine. The determination of thiocholine is accom= plished by a continuous reaction of the thiol with 5:5-dithiobis-2-nitrobenzoate. This reaction produces a yellow anion of 5-thio-2-nitro-benzoic acid, permitting a continuous recording of the rate of the reaction in a spectrophotometer at a wavelength of 412nm.

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The acetylcholinesterase activity of the salivary secretion by this method was performed as follows : the salivary secretion (20 - 50 µl) was introduced into a spectrophotometer cuvette contain= ing 30 µl of a 0,075M acetylthiocholine iodide solution in water, 100 µl of 0,01M DTNB solution, in 0,1M sodium phosphate buffer at pH 7 and a quantity of 0,1M sodium phosphate buffer at pH 8, so as to give a final volume of 3 ml. The control contained the above solutions, except for the salivary secretion which was substituted with distilled water. The appearance of the thiol was recorded automatically at 25 ^{0}C with a Beckman DK2A ratio recording spectrophotometer. The increase in optical density at 412nm over a time period of 5 minutes was read from the recording and the ac= tivity expressed as µmoles substrate hydrolyzed by 1 ml salivary secretion per minute. This value was found to be 9,7.

To test for the participation of free sulfhydryl groups present in the salivary secretion (179), the assay was performed as described above but omitting the sub= strate. The result showed no such partici= pation.

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The acetylcholinesterase activity of the peaks obtained from the first Sephadex G100 column (Fig 3(c)) were determined using up to 100 µg of the fractions in the assay mixtures. Activity was found in Peak I (specific activity ; 0,4). A single peak, with specific activity of 0,1 emerged at the void volume after chromatography of this peak on a sephadex G200 column (2,5 x 40 cm) with distilled water as eluant.

The influence of various substrate con= centrations and pH on the activity of the first peak obtained from the Sephadex G100 column (Fig 3(c)) was investigated. The results are shown in Figs 26 & 27.

2.6 INVESTIGATION INTO THE HEMOLYTIC ACTIVITY OF THE ORAL SECRETION

Observations made by Howell and Pienaar (22) re= garding the symptoms produced by the oral secre= tion in experimental animals (See Section 2.2.2.1), suggest that hemorrhagic conditions occur after introduction of the secretion. Since this syndrome could be the result of hemolysis, the hemolytic activity of the secretion was investigated.

For this study the method of Hessinger and Len= hoff (180) was employed. The hemolysis reac= tions were performed on washed sheep red blood cells. Blood was collected directly in centri= fuge tubes on ice and centrifuged for 5 minutes

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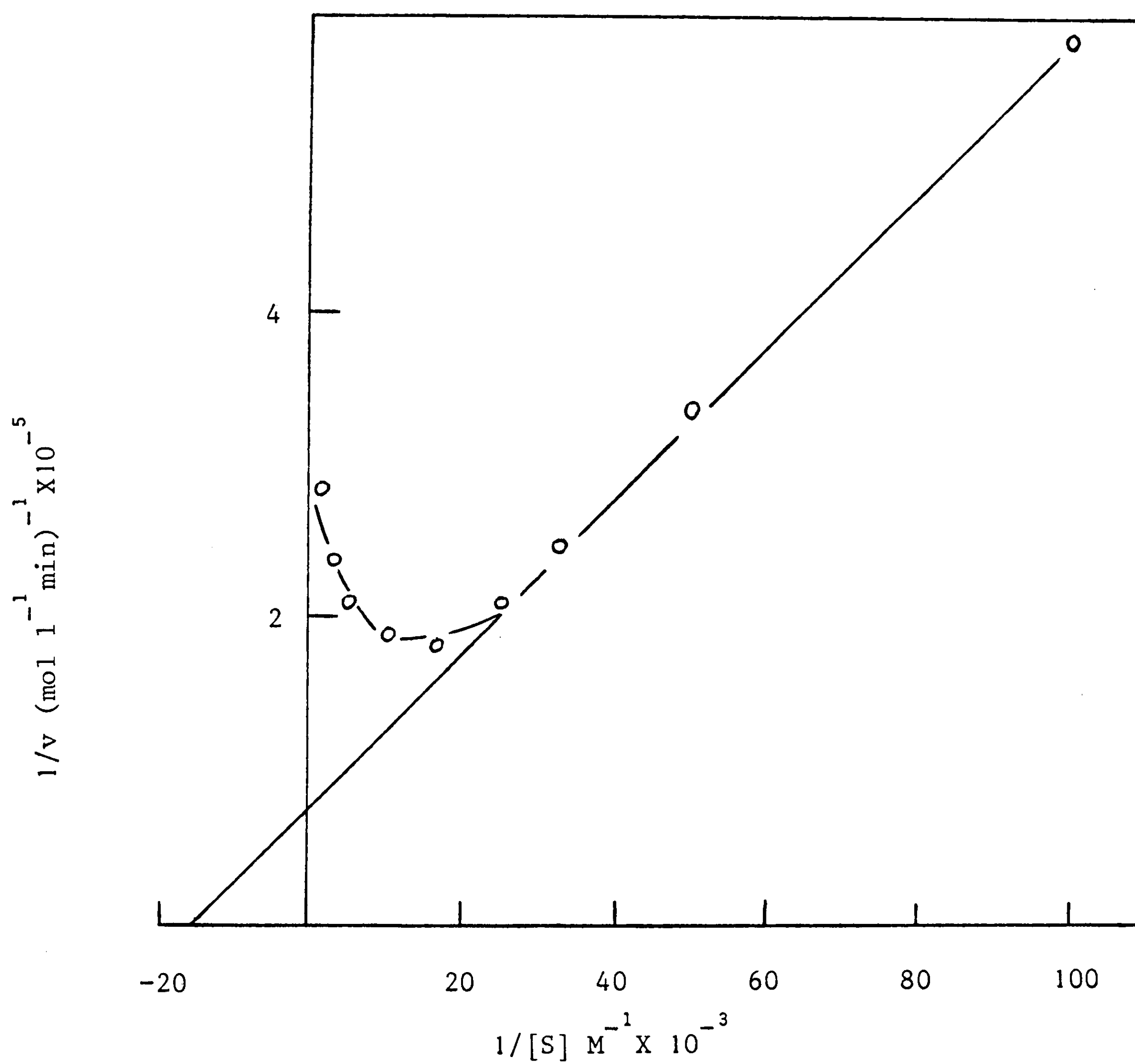

Fig 26 Lineweaver - Burke plot showing the effect of substrate concentration [S] on the acetylcholinesterase activity of Fraction I (Fig 3 (c)) at a concentration of 0,02 mg ml^{-1} assay mixture. The K_m value is 62,5 μmol l^{-1}

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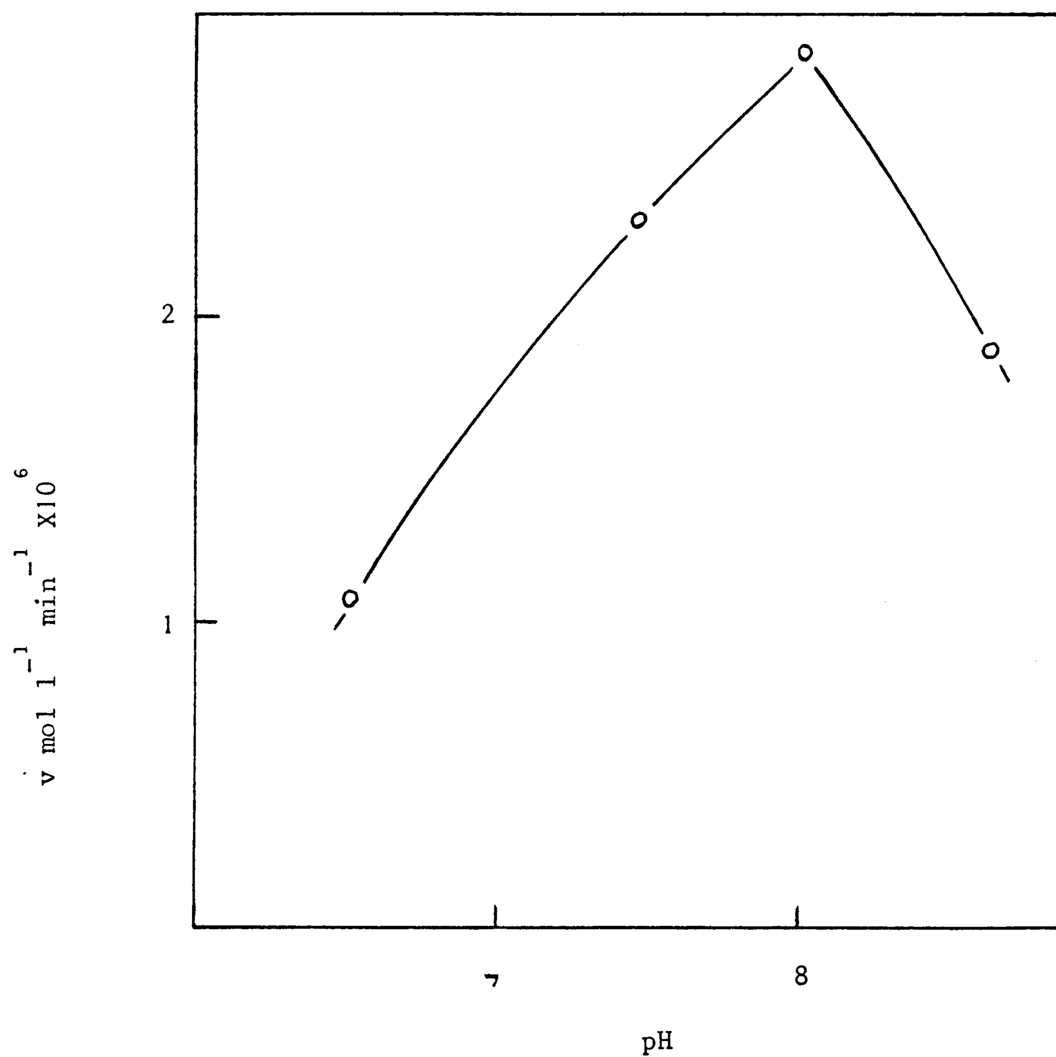

Fig 27 Effect of pH on the acetylcholinesterase activity of Fraction I (Fig 3(c)) at a concentration of 0,013 mg ml^{-1} assay mixture. The activity deter= minations were performed as described in the text with 0,1M sodium phosphate buffer at pH 6,5; 7,4 and 8 and with 0,1M Tris buffer at pH 8,6.

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at 1000 g at 4 ^{0}C. The serum was removed by aspi= ration and the pellet containing the red blood cells, suspended in 10 times its volume of an isotonic saline-Tris solution at pH 7,4 (144 mM NaCl; 10 mM Tris-HCl). The washing procedure was repeated twice. The hemolysis assays were performed with 20 ml of 0,6% (v/v) red cell suspen= sions in a solution of the following composition: 138 mM NaCl, 10 mM Tris, 3 mM KCl, and 2 mM $CaCl_2$, pH 7,4 and containing from 1 to 100 µl of salivary secretion. (The supernatant, obtained as described below of a 0,6% (v/v) red cell suspension in dis= tilled water, which resulted in 100% lysis, had an optical density of 0,93 units measured at a wavelength of 570nm).

The assay mixtures were equilibrated at 30 ^{0}C and at various time intervals, 3 ml aliquots were withdrawn and centrifuged immediately at 10 000 g for 5 min at 5 ^{0}C. The optical density of the supernatant was determined at a wavelength of 570nm. The result showed that no hemolysis had occurred even after 24 hours with 100 µl salivary secretion

2.7 SOME OBSERVATIONS REGARDING THE SALIVARY SECRETION OF THE BONTBOSLUIS, *Amblyomma hebraeum*

2.7.1 Introduction

The bontbosluis, *Amblyomma hebraeum* is found in southern Africa in the Bushveld north of the Magaliesberg, Botswana, the southern parts of Rhodesia, the eastern

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Transvaal, Swaziland, Natal and the eastern Cape Province, especially in the district of Mosselbaai along the Klein-Brak river (181). Apart from high grass, trees are essential for their existence (181).
The most preferable hosts are cattle and wild buck. The larva and nymph also feed on numerous small animals, especially on rabbits. In contrast to the Sand tampan, the bontbosluis is a slow feeder and may feed up to three weeks on the hosts. The starvation-survival time for larva is 9 months, for the nymph, 6 months and for the adult tick, 14 months (181).
The bontbosluis, also known as the hart=water tick, is the transmitter of Cowdria ruminantium (Rickettsia ruminan=tium), causing hartwater in cattle, sheep and buck (5, 182). The tick is also the transmitter of tick fever (Rickettsia rickettsi pyperi) (181).
During the course of the study of the salivary secretion of the Sand tampan, a limited quantity (2 ml) of the salivary secretion of the bontbosluis became available (183). Some observations re=garding the biochemical composition of this secretion (184) and a comparison

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of the results with those obtained from the secretion of the Sand tampan is re= ported in this section.

2.7.2 Gel permeation chromatography of the secre= tion.

The clear salivary secretion (2 ml) was submitted to gel chromatography. Upward flow through a column (36 x 2,5 cm) of Sephadex G100 at a flow rate of 21 ml h^{-1} was used as the initial separation step with distilled water as eluant. Fractions of 2,6 ml were collected and monitored at a wavelength of 280nm in a Beckman DK2A spectrophotometer. The separation pattern is shown in Fig 28 (a). In Fig 28 (b) the separation pattern after chromatography of the salivary secretion of the Sand tampan on a similar column is shown for comparison (See Fig 3 (c)).

Fraction II obtained from the Sephadex G100 column (Fig 28 (a)) was subsequently submitted to chromatography on a Bio-Rad P10 column. The column dimensions, flow rate and the collection and moni= toring of the fractions were similar to those described for the Sephadex G100 column. The elution diagram is shown in Fig 29.

2.7.3 Total free amino acids in the secretion

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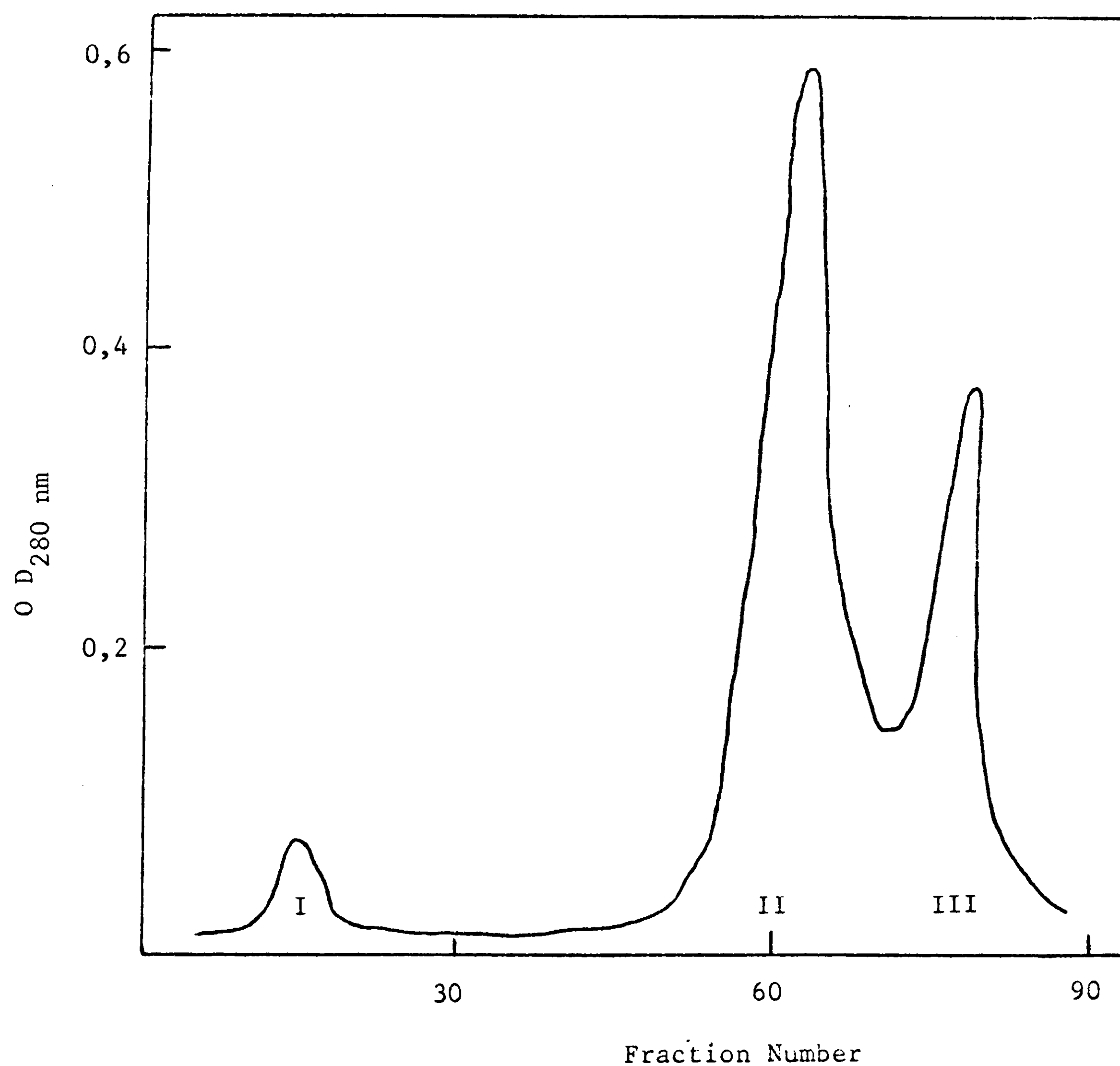

Fig 28(a) Gel chromatography of the oral secretion of the bontbosluis on Sephadex G100. Details of the proce= dure are described in the text.

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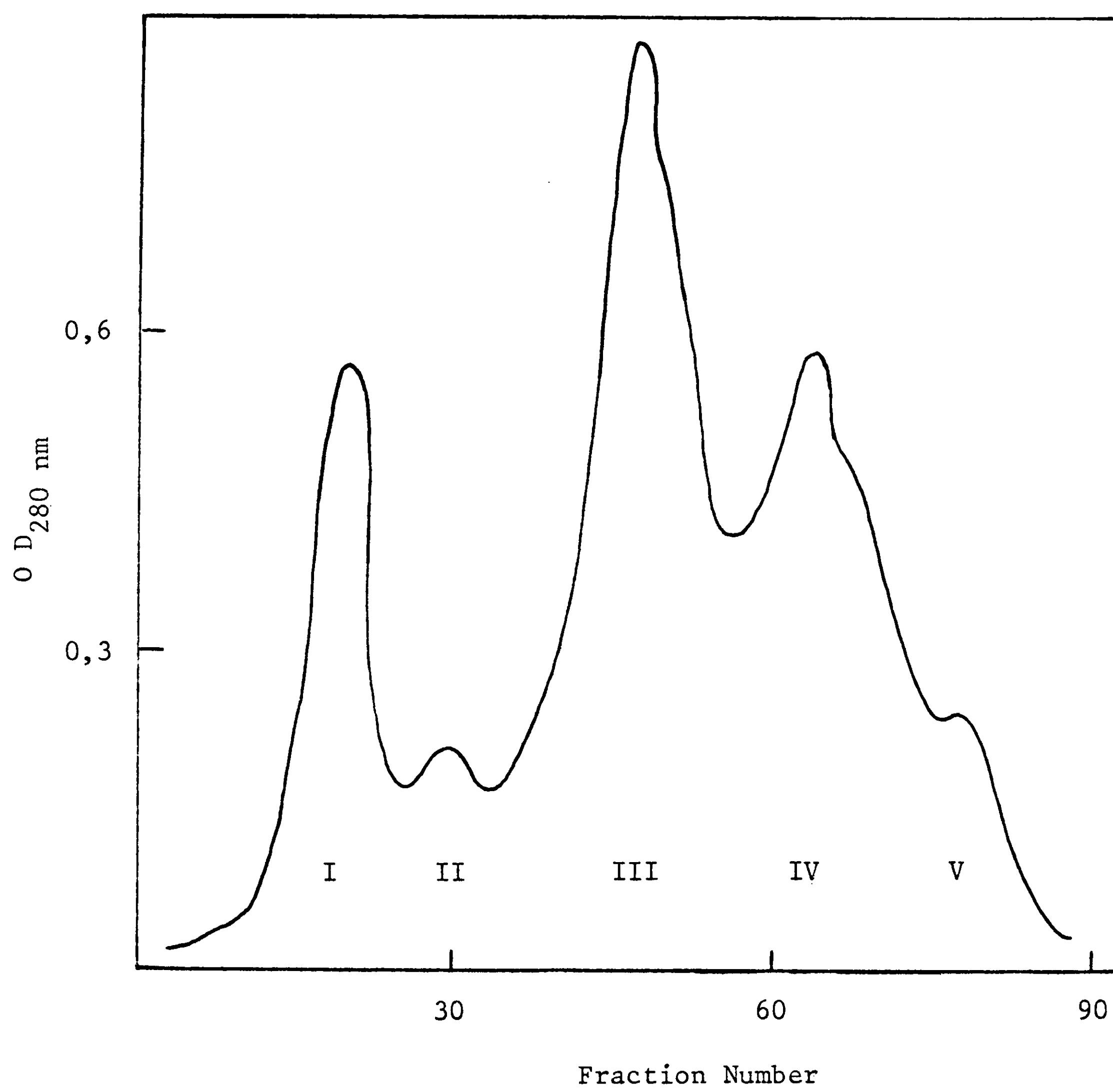

Fig 28(b) Gel chromatography of the oral secretion of the Sand tampan on Sephadex G100. Details of the procedure are described in the text.

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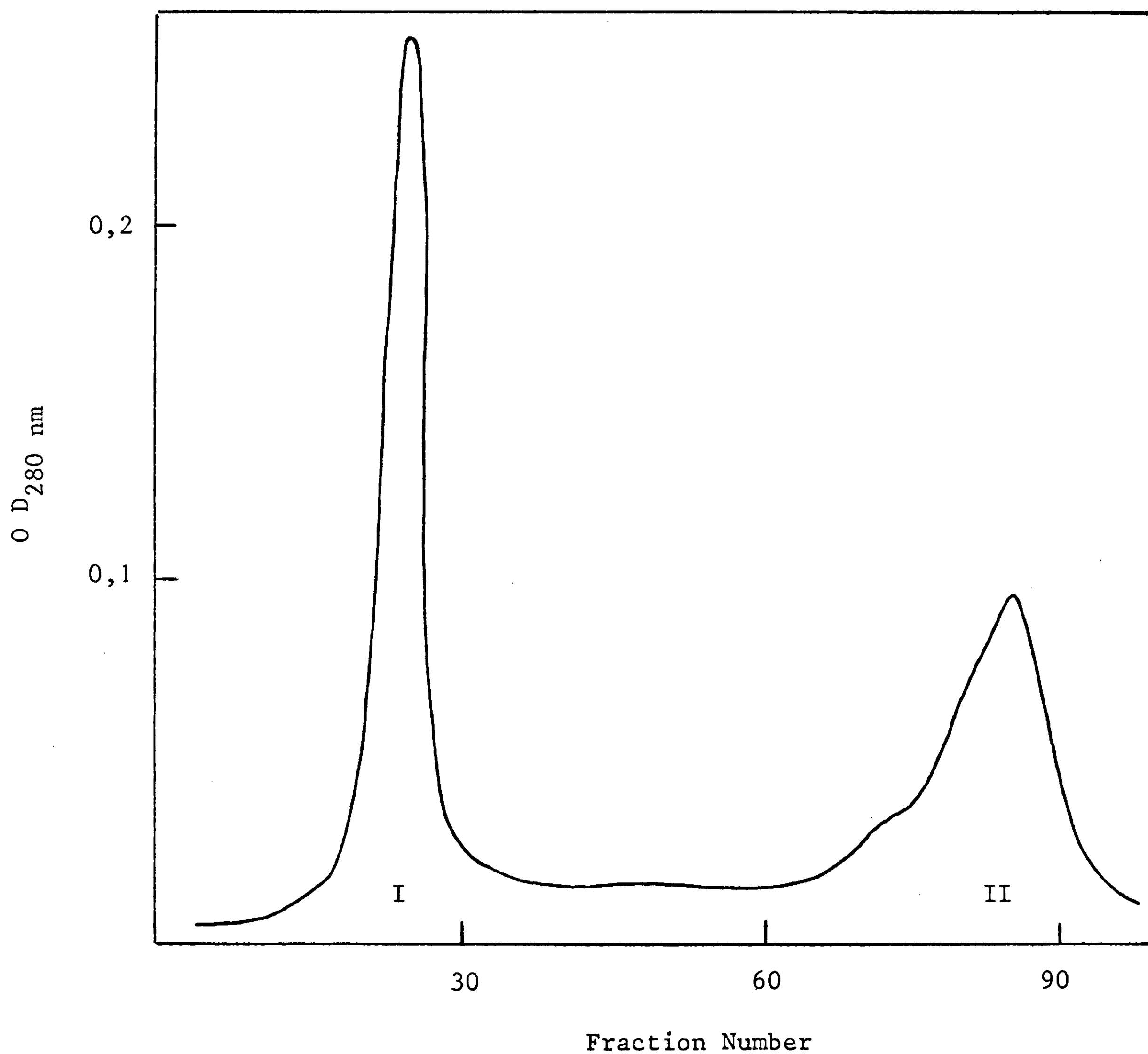

Fig 29 Gel chromatography of Fraction II obtained from the Sephadex G100 column (Fig 28(a)) on a Bio-Rad P10 column (36 x 2,5 cm). Eluant : distilled water. Flow rate : 21 ml h^{-1} and fractions of 2,6 ml collected.

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Determination of the amino acids present in the unhydrolyzed fraction III (Fig 28 (a)) and fraction II (Fig 29) was achieved with a Beckman Model 120B amino acid analyzer as described in Section 2.2.3.5. The sum of the individual amino acid concentrations in these fractions were considered to represent the total of each free amino acid present in the salivary secretion. The results are shown in Table 17. The total free amino acids present in the oral salivary secretion of the Sand tampan and human saliva (185) are shown for comparison.

2.7.4. Characterization of Fraction I obtained from the Bio-Rad P10 column (Fig 29)

This fraction showed weak hyaluronidase activity which was determined as described in Section 2.5.2. A reduction of 15% in turbidity of the hyaluronic acid-egg albumin complex resulted after 0,036 mg of the fraction had acted on 0,28 mg of substrate for 45 min at 37 ^{0}C.

The amino acid composition of this fraction was determined as described in Section 2.4.2. The results are shown in Table 18 together with the composition of testicular hyaluronidase (186, 187).

The molecular mass of this fraction, de=

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TABLE 17 FREE AMINO ACIDS PRESENT IN THE SALIVARY SECRETIONS OF *A. hebraeum*, *O. savignyi* AND HUMANS ($\mu g\ ml^{-1}$)

Amino Acid	*A. hebraeum*	*O. savignyi*	Human (Woldring, 1955; ref. 185)
Lysine	36,7	98,9	7,7
Histidine	28,9	19,6	8,1
Arginine	70,2	0	1,9
Aspartic acid	13,3	trace	1,3
Threonine	0	42,6	2,6
Serine	731,6	4,6	2,6
Glutamic acid	32,7	trace	3,8
Proline	25,3	trace	5,1
Glycine	137,4	4,5	9,1
Alanine	81,7	17,6	4,6
Valine	139,1	68,3	1,6
Isoleucine	24,6	34,6	4,4
Leucine	52,2	77,9	3,4
Tyrosine	35,9	63,8	4,8
Phenylalanine	24,1	55,4	3,9
Taurine	119,6	44,5	4,1
TOTAL	1553,3	532,3	69,0

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TABLE 18 AMINO ACID COMPOSITION OF A HYALURONIDASE ACTIVE FRACTION FROM THE SALIVARY SECRETION OF *A. hebraeum* COMPARED TO HYALURONIDASE FROM TESTICULAR TISSUE (g amino acid residue/100 g enzyme).

Residue	*A. hebraeum*	Testicular tissue (Borders & Raftery, 1968; ref 186)	Testicular tissue (Brunish & Högberg, 1960; ref 187)
Lysine	4,68	4,77	4,21
Histidine	3,77	1,82	1,51
Arginine	3,59	4,37	3,39
Aspartic acid	7,19	7,83	7,78
Threonine	3,13	3,34	2,84
Serine	4,38	2,87	3,74
Glutamic acid	8,00	6,55	6,36
Proline	3,73	3,58	3,27
Glycine	2,54	2,13	2,09
Alanine	2,63	2,49	2,55
Valine	3,51	4,37	4,20
Isoleucine	2,26	2,87	2,73
Leucine	5,03	6,13	5,66
Tyrosine	3,43	3,98	3,22
Phenylalanine	3,61	3,61	3,29

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termined by sedimentation equilibrium centrifugation by means of the ultravio= let photoelectric scanner as described in Section 2.4.3 was found to be 9680, assuming a partial specific volume of 0,725 ml mg^{-1}. The plot of log (O D) against r^2 is shown in Fig 30 which in= dicates a homogeneous preparation.

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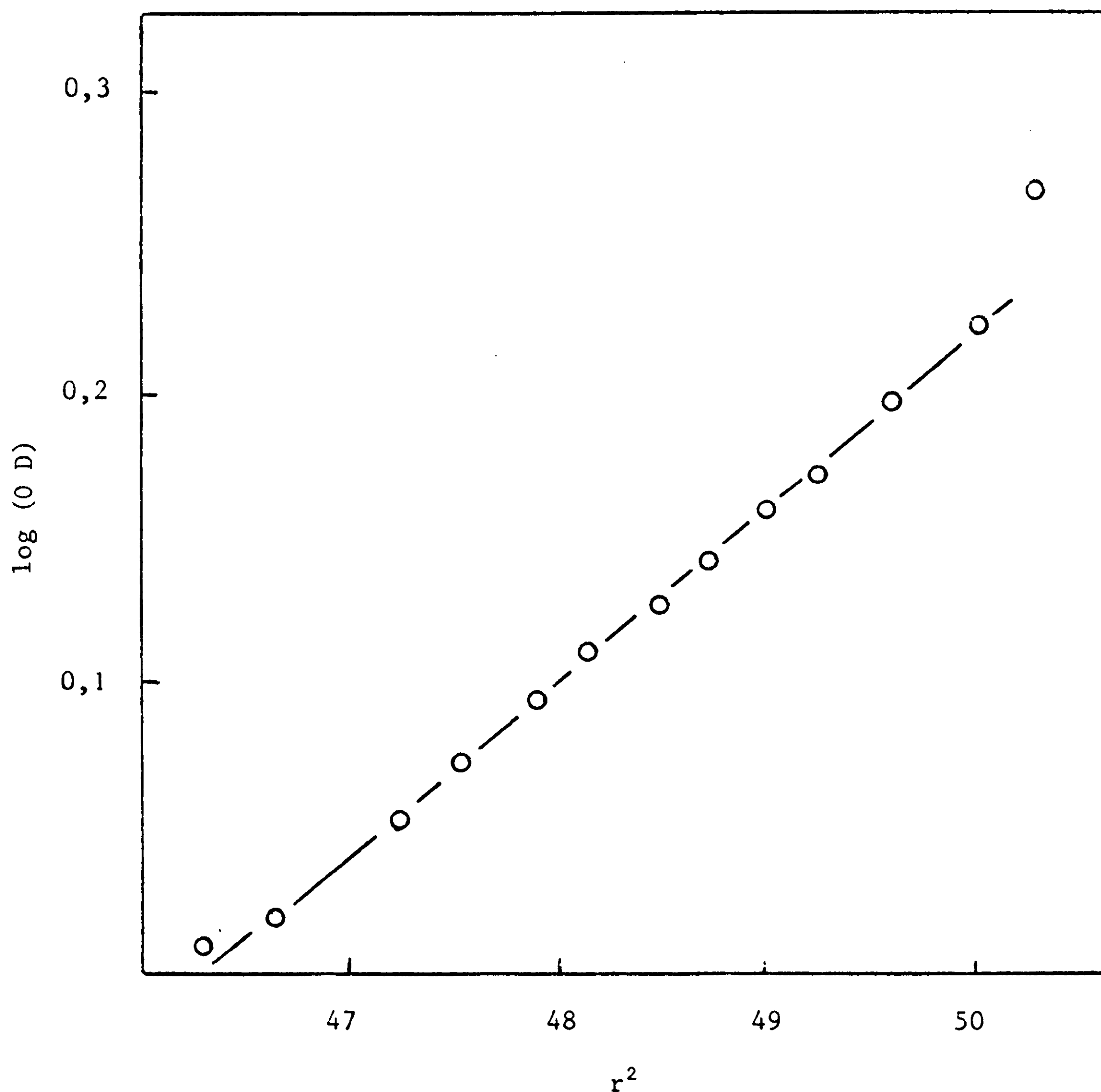

Fig 30 Plot of log (O D) versus r^2 employed for the molecular mass determination of the hyaluronidase activity of Fraction I (Fig 29) by sedimentation equilibrium centrifugation. Details of the determination are described in Section 2.4.3.

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CHAPTER III

DISCUSSION AND CONCLUSIONS

The collection of *Ornithodoros savignyi* ticks and their oral salivary secretion by methods described by Nevill (20) and Howell (4) proved to be satisfactory. For obvious reasons the development of an automatic pro=cedure for the collection of the salivary secretion to obviate the need for injection of a stimulant and placing a capillary tube over the mouthparts of each individual tick, should receive more attention.

Salivary secretion of the Sand tampan could possibly be obtained by infrared heat, electrical stimulation or by the topical application of a stimulant. For the success of the latter method more knowledge concerning the composition of the Sand tampan would be helpful especially for the se=lection of a suitable solvent for the stimulant. The observa=tions of Barker *et al*. (23) regarding variations in the electrolytic, protein and amino acid composition of tick salivary secretions, collected by infrared heat, pilocarpine injection and electrical stimulation should, however, be borne in mind and need to be further investigated.

The oral salivary secretion of the Sand tampan is a complex mixture of numerous protein-like components. This is evident from the elution patterns of gel permeation and ion exchange chromatography, microzone electrophoresis and isoelectric focusing of the oral secretion. In striking contrast, are the rather simple protein patterns of the corresponding secretions obtained from the bontbosluis

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and the cattle tick (188).

Of considerable interest is the finding that the salivary secretions of the Sand tampan and bontbosluis show definite variations with respect to their free amino acid content. Furthermore, when the free amino acids of human saliva (Table 17) are compared, it is evident that these two tick species have an appreciable larger amount of these acids in their saliva. It is a well established fact that insect hemolymph contains extraordinary high con= centrations of free amino cids. Values of up to 20 mg ml^{-1} have been reported (189). This high free amino acid con= tent may be considered as one of the many unique aspects of the biochemistry of insects (14). It may well be that this finding also applies to the free amino acids of the salivary secretion of ticks. In the hemolymph the composi= tion of these acids vary widely, not only from one species to another but also within the same species. Temperature, diet and stage of development affect the composition (14). Nevertheless, certain species can be differentiated from others according to the concentration of some amino acids.

In spite of the degree of variablility within the same species, some attempts have been made to express the amino-acidemia in terms of metabolism. For example the free cysteine content of tick body fluids is of interest since this amino acid may participate in the metabolism of arsenicals. Stocken and Thompson (190) have clearly shown that one mode of action of arsenicals is their re= action with sulfhydryl compounds. Whitehead (191) has indeed shown that arsenic resistant B. decoloratus ticks contain greater amounts of sulfhydryl compounds than sus=

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ceptible ticks. The close relationship of tyrosine in the hemolymph to the O-Quinones in the cuticle is noteworthy (192). A more complete knowledge of the cuticle composition and a detailed study of the biosynthesis of these components is of particular interest when penetrant carriers, which enhance acaricide penetration through the cuticle are considered. Most likely other significant correlations be= tween amino acids in the body fluids and metabolism may emerge in the future.

When considering the free amino acids present in the salivary secretion of A. hebraeum, it is evident that the serine content is particulary high, amounting to almost 50% of the total amino acid content. It is almost certain that the peak, emerging at the serine position during amino acid analysis is contaminated with other amino acids, most like= ly glutamine and asparagine. These latter two amino acids are of importance in insect metabolism since they serve as effective trapping agents of ammonia, released after deamina= tion (193). The deamination of amino acids to provide sub= strates for the tricarboxylic acid cycle may be an important function of free amino acids in insect tissues. Wintering= ham et al. (194) have shown that diisopropylphosphofluoridate causes an increase in the free glutamine concentration in the adult housefly. This glutamine accumulation may indicate fatal biochemical lesions in addition to that of acetylcholin= esterase inhibition. The study of the influence of acarici= des on the amino acid composition of body fluids may prove to be useful in the elucidation of their precise mode of action.

A relatively simple procedure for the isolation of a toxic fraction present in the oral salivary secretion of the

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Sand tampan was developed during the course of this in= vestigation. The most important modification of previous isolation methods (9), was the introduction of distilled water as eluant in the gel permeation steps. It should be noticed that distilled water was employed as eluant only after it was established without doubt that this eluant had no detrimental effect on the toxic fraction. Another important modification was the elimination of the ion exchange chromatography step. These modifications eliminated isolation steps, during which loss of toxic activity may occur, particularly during dialysis as described previously (9). It should be stressed that it was proven without doubt that the simpler isolation procedure resulted in a toxic component identical to that obtained by original methods (9) and with the profitable result of obtaining a substantial higher yield. The LD_{50} of this fraction was not determined. However, subcutaneous injection of 0,4 mg into albino mice, weighing 10 g resulted in death after about 90 minutes.

With the inherent difficulties associated with defining a homogeneous biological acitivity in mind, it may be stated that the toxic component isolated by the methods described in this thesis was found to be homogeneous as shown by chromatographic, electrophoretic, ultracentri= fugal and amino acid sequence analyses.

Valuable information regarding the structure of the toxin was obtained from the amino acid analysis data, electrophoretic behaviour of the toxin, ultracentrifugal investigations of the toxin under various conditions, carbo= hydrate analysis and amino acid sequence determinations.

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The amino acid composition of the toxin as reported in this thesis cannot be regarded as being accurate since it is expected that carbohydrate and amino acid residues had interacted during hydrolysis of the toxin. These interactions result in disintegrations of amino acids, thus diminishing their yield.

The molecular mass of the toxin, determined by sedimentation equilibrium centrifugation, was found to be dependent on the sample concentration and pH of the sol= vent. Extrapolation of the molecular mass _versus_ concen= tration plot (Fig 13(b)) to zero concentration at a sol= vent pH of 7,9 shows a molecular mass of 6800. This value corresponds well with the minimal molecular mass of 6346 calculated from the amino acid composition and is in fair agreement with the value of 7800 obtained by sedi= mentation equilibrium centrifugation, of the toxin after exposure to 8M urea. At relatively high sample concentra= tions at pH 7,9 the molecular mass of the toxin was found to be approximately 15000 according to ultracentrifugal methods.

The molecular mass determinations reveal that the toxin in the salivary secretion most probably, exists as a dimer since the average pH of the secretion is 7,9 and the toxin is present in relatively high concentration. When introduced into the tissues of a host, however, the toxin is diluted and with only a slight change in the pH of its new environment it most likely dissociates into monomers.

Of interest is the influence of alkali and alkali combined with sulfite treatments on the molecular mass of the toxin. These results together with other observations

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(increase of optical density at 241nm, reduction of threonine residues and an increase in the cysteic acid residues after treatment with alkali and sulfite, together with amino acid and carbohydrate analysis data) indicate that the toxin is a glycoprotein. Furthermore it is evident that the carbohydrate moiety is attached to the protein chain via an O-glycosidic linkage. Removal of the carbohydrate moiety results in the complete loss of toxicity.

Calculation of the minimal molecular mass of the protein portion from amino acid analysis data yields a value of 4652 which is in a close agreement with the value of 4492 calculated from sedimentation equilibrium analysis. This latter value corresponds with the protein portion of the toxin after β-elimination of the carbohydrate chain by alkaline-sulfite treatment. The molecular mass of 2422 obtained after alkali treatment (in the absence of sulfite) may be explained by the observations of Berg=mann and Grafe (97) who showed that dehydropeptides, which are produced after β-elimination are unstable.

Amino acid analysis of the toxin after dithio=threitol reduction and subsequent iodoacetic acid treat=ment and iodoacetic acid treatment without prior reduction show that the toxin contains no free sulfhydryl groups.

Amino acid analysis, molecular mass, isoelectric point, and amino acid sequence determinations of the first approximately 30 amino acids has revealed that the toxic and non-toxic components show many similarities. A more complete study of these two fractions, expecially with respect to the elucidation of their entire amino acid sequence and a comparison of their carbohydrate moieties is of paramount importance to evaluate the precise

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homology between these two proteins. These two components provide an ideal model for the study of a correlation between protein structure and function.

An interesting amino acid sequence heterogeneity was observed at residue 1 of the toxic component. Both glycine and aspartic acid were found. Of these two residues, glycine was obtained in the highest yield. The aspartic acid yield was approximately equal to that ob= tained for this amino acid at residue 1 in the non-toxic component. Contamination of the non-toxic with the toxic component can be excluded as the cause of the heterogeneity since no amino acids of the former component were observed at corresponding positions of the latter where these two components show differences in the sequence (for example at positions 5, 6, 15, 18 and 26). The well established genetic code (195) reveals that this heterogeneity cannot be explained by the mutation of a single nucleotide.

Although the efficiencies of the sequence deter= minations were found to be above 90%, the initial yields were low. This may be explained by the fact that aspartic acid occurs in the first residue in both the toxic and non-toxic components (in the former component, together with glycine). Aspartic acid is known to undergo a $\alpha \rightarrow \beta$ shift when in the N-terminal position. This shift may occur under mild acid conditions (144). These conditions were encountered during the isolation of the component after S-carboxymethylation (See Section 2.4.7).

At several residue positions in the amino acid sequence of both components no amino acids could be iden= tified. At some of these positions this might have been due to attached carbohydrates at threonine residues.

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To circumvent this difficulty it is suggested that the sequence analysis be performed on fractions after ex= posure to mild alkali treatment in the presence of sodium borohydride and palladium chloride (196). This procedure produces α-amino-butyric acid which may be detected as the PTH-derivative by the methods described in Section 2.4.9

An investigation into the effects of the salivary secretion in host and experimental animals revealed a complex picture (22) in which the disturbance of only one biological system by the components of the secretion most probably affects closely integrated systems. The sequence of events which result in the death of the animals is therefore, difficult to analyse. Furthermore, it was found that a considerable loss in potency of the toxin occurred during its isolation which may indicate that other components present in the secretion contribute to its toxic effect. For these reasons it was soon realised that an analysis of the secretion as a whole should be ventured upon in order to enventually obtain a clear picture of the biochemical basis of the syndromes produced by the secretion. In addition, the information obtained by such an analysis could be useful to explain host and pathogen specificity of ticks in general. In working towards this goal some biochemical activities of the secretion were investigated.

The hyaluronidase, proteolytic and hemolytic ac= tivities in the secretion were investigated as it was reasoned that they may have a bearing on the hemorrhagic conditions produced by the secretion in host and experi= mental animals.

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Hyaluronidase, apart from acting as a "spreading agent" in snake, bee and scorpion venoms, many invasive bacteria and bloodsucking insects (197), by lowering connective tissue barriers, has a profound effect on the properties of the capillary wall. The enzyme is known to cause serious symptoms in animals. Patechial hemorrhages caused by the enzyme has been described by Chambers and Zweifach (198). The mechanism by which the enzyme may effect capillary permeability and capil= lary fragility has been described by Copley (199). Hyaluronidases from different biological origins show marked differences with respect to specific activity, substrate specificity, mechanism of action, end products produced, chemical composition and physical characteris= tics (197). These differences may have a decisive effect on the symptoms produced by these various enzymes.

Hyaluronidase activity was found in the salivary secretion of the Sand tampan. In addition, this enzyme was detected in the salivary secretion of the bontbosluis. The enzyme in the latter secretion was isolated in a pure form and partially characterized. It shows some similari= ties with the testicular enzyme as far as the amino acid composition is concerned. A comparison of the molecular mass is impossible however, since values of between 11000 and 61000 have been reported for the bovine testicular enzyme (186, 187). The enzyme of the Sand tampan and the bontbosluis show a low actvity. It should be mentioned however that optimal conditions for activity measurements were not determined.

The proteolytic activity of the salivary secretion

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was found to be specific in nature. It was shown that the secretion hydrolyzed typical synthetic trypsin sub= strates but chymotrypsin substrates were not hydrolyzed. Of interest is the fact that pepsin-like activity was found.

An inhibitor of the protease activity was detec= ted in the secretion which had no effect on pancreatic trypsin. This inhibitor is most probably only effective in the salivary secretion and is most likely inactivated by the tissues of the host

Although no hemolytic activity was shown to be present it should be borne in mind that sheep erythrocytes were used in the investigations and in the absence of added lecithin. Further investigations should be made employing erythrocytes from various animal sources in both the absence and presence of lecithin. This suggestion is made since erythrocyte osmotic fragility varies greatly amongst animal species (200). Furthermore, it is possible that a lytic effect of a toxin may be the result of a specific hydrolyzing action on lecithin present in the membrane of erythrocytes. When this substrate is not available to the toxin because of, for example, steric hindrance, no hemolysis is possible (201). By adding lecithin, lysolecithin may be formed which is the actual lytic agent.

The acetylcholinesterase activity present in the secretion is of interest, especially when acaricides, which act as inhibitors of this enzyme are considered. The enzyme is present in very low concentrations in the secretion and shows higher activity with acetylthiocholine than with acetylcholine bromide as substrate.

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The results reported in this thesis as well as results that will be obtained from future investigations into the salivary secretions of other species of ticks may eventually be correlated with distinct overall characteristic features of various species of ticks; un=doubtedly more knowledge is essential for a better and true understanding of tick, host and pathogen inter-relation=ships, host immunization, chemotherapy and vector control.

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"Biochemical investigation into the toxic salivary secretion of the tick, Ornithodoros savignyi Audouin (1827)"

by

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Thesis submitted for the D Sc(Agric) degree

SUMMARY

Ticks, including the Sand tampan Ornithodoros savignyi, pre= sent important economic problems over a large area in the Republic of South Africa (S 1). In all known diseases of which ticks are the vectors, the causal agents (protozoa, rickettsias, anaplasms, viruses, spirochaetes and bacteria) have been identified with the exception of tick toxicoses in which pathogenic agents are evidently not involved (S 2). Thus, the causal agents associated with tick toxicoses caused by the Sand tampan have not been identi= fied. These causal agents appear to develop only in the invertebrate host (the ticks) and their timely removal from the vertebrate hosts, is followed by a subsidence of the symptoms. Recovered animals develop a durable im= munity (S 3).

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The observations regarding tick paralysis and sweating sickness are most likely also applicable to tick toxicoses caused by the Sand tampan.

Claims of toxins present in the salivary secretions of ticks have been made (S 1). Evidence supporting these claims are presented in this thesis. Information is pro= vided concerning the chemical composition of the salivary secretion and an isolation procedure by means of which a toxic component present in the secretion may be obtained in a pure form. In addition some chemical components and activities in the oral secretion of the bontbosluis, Amblyomma hebraeum, were investigated and the results reported.

Sand tampan ticks used for the investigations were collected in the North West Cape Kalahari with traps baited with solidified carbon dioxide (S 5). Oral se= cretions of the ticks were obtained by parasympathetic stimulation as described by Howell (S 6). Some pro= perties of the secretion are shown in Table S 1.

The enzymatic activities undoubtedly enhance the entry of the toxin into the tissues of the host.

Isolation of the toxic activity was achieved by means of gel and ion exchange chromatography and isoelec= tric focusing. The toxin was found to be homogeneous according to ultracentrifugal and gel-electrophorectic techniques and isoelectric focusing. Some characteristics of the toxic component are presented in Table S 2.

Removal of the carbohydrate moiety or S-carboxy= methylation of the toxin results in a complete loss of toxic activity. A non-toxic glycopeptide which showed

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most of the above characteristics was found in the salivary secretion. It differed slightly from the toxin with respect to the isoelectric point (0,3 units lower) and amino acid sequence (Table S 3). A detailed structural comparison of these two glycopeptides could aid in the elucidation of the structural requirements essential for toxicity.

The syndromes produced by the salivary secretion (S 25) indicate that the toxin may exert its effect via increased capillary permeability or fragility. Since a method has been developed for the purification of the toxin and its chemical nature has been partially described, the biochemical lesion(s) can now be investigated.

The total free amino acids present in the salivary secretion of the bontbosluis, Amblyomma hebraeum was deter= mined. A total of 1553 μg of amino acids per ml secretion was found. This value is approximately 3 times higher than the value for the Sand tampan and approximately 20 times higher than for human saliva.

The salivary secretion of this tick was fractionated by gel chromatography. One of the fractions showed weak hyaluronidase activity and was found to be homogeneous according to sedimentation equilibrium centrifugation. The molecular mass, determined by the latter method was found to be 9680. The amino acid composition of the fraction revealed, with a few exceptions, similarity with that of hyaluronidase isolated from bovine testicular tissue.

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TABLE S 1 PROPERTIES OF THE SALIVARY SECRETION OF *C. savignyi*.

Properties of the salivary secretion		Reference to method employ= ed
Yield	48 nl mg^{-1} tick body mass	S 6
pH	7,9	
Absorption maximum	at 276 nm	
Total nitrogen	3,6 mg ml^{-1}	S 7
Non-protein nitrogen	0,5 mg ml^{-1}	S 8
Total amino acids	21,2 mg ml^{-1}	S 9
Free amino acids	0,55 mg ml^{-1}	S 8
Toxicity (LD_{50} for mice)	0,21 ml kg^{-1} body mass	S 10
Heat stability of toxic activi= ty	stable to 82 ^{0}C	
Cholinesterase activity	9,7 mol substrate hydrolyzed min^{-1} ml^{-1}	S 11
Proteolytic activity as deter= mined with the following sub= strates:		
(i) Casein	2,8X10^{-2} Kunitz Units	S 12
(ii) Benzoyl-L-arginine ethyl ester	3,3 μmol substrate hydrolyzed min^{-1} ml^{-1}	S 13
(iii) Acetyl-L-tyrosine ethyl ester	no activity	S 14
(iv) Benzoyl-L-arginine-p-nitroanilide	0,2 μmol substrate hydrolyzed min^{-1} ml^{-1}	S 15
(v) Acetyl phenylalanyl-L-diiodotyrosine	0,05 μmol substrate hydrolyzed min^{-1} ml^{-1}	S 16
Hyaluronidase activity	71,4 Units ml^{-1}	S 17

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TABLE S 2 PROPERTIES OF THE TOXIC COMPONENT

Properties of the toxic component		Reference to method employ= ed.
Yield	3-4 mg ml^{-1} secretion	S 18
Toxicity (quantity injected subcutaneously into 10 g mice)	0,4 mg results in death after 90 min	S 18
Absorption maximum	278 nm	
Nitrogen content	14,9%	S 7
Isoelectric point	5,1	S 7
Amino acid content	72,1%	S 9
Total carbohydrate content	11,3%	S 19
Sialic acid content	0,9%	S 20
Galactosamine and glucosamine content	absent	S 21
Carbohydrate - peptide linkage	O-glycosidic type in= volving serine or threonine	S 22
Molecular mass	6800	S 23
Molecular mass of peptide	4652	S 22, S23
Molecular mass of carbohy= drate moiety	(6800-4652)=2148	
Enzymatic activity with re= spect to substrates listed in Table S 1	no activity	See Table S 1

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TABLE S 3 PARTIAL AMINO ACID SEQUENCE OF THE TOXIC AND CLOSELY RELATED NON-TOXIC COMPONENT DETERMINED BY THE METHOD OF EDMAN (S 24)

Component		Sequence
		1 … 10
Toxic component	*(TC)*	*Gly-CMCys-Pro-Pro-Gly-Val-Pro-Thr-Arg-Ala-*
Non-toxic component	(NTC)	Asp-CMCys-Pro-Pro-Thr-Lys-Pro-Thr-Arg-Ala-
		11 … 20
	(TC)	*Tyr——Val-Ala-Phe-Val-Glu-Gly- - -Gly-Ala-*
	(NTC)	Tyr——Val-Ala-Phe- - -Glu-Gly-Glu- - -Ala-
		21 … 30
	(TC)	*Tyr——Leu-Ile-Val-Val-Thr-Leu- - -Leu- - -*
	(NTC)	Tyr——Leu-Ile-Val-Val-Ser- - -Asp-Leu- - -
		31
	(TC)	*Ala→Leu-*
	(NTC)	Ala-

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'n Biochemiese ondersoek van die toksiese speekselagtige sekresie van die bosluis, Ornithodoros savignyi Audouin (1827).

deur

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Proefskrif ingedien vir die D Sc(Agric)-graad

SAMEVATTING

Bosluise, insluitende die Sandtampan, Ornithodoros savignyi, skep ernstige ekonomiese probleme in groot dele van die Republiek van Suid-Afrika (S 1). In alle bekende siektetoestande veroorsaak deur bosluise is die veroorsa= kende agente (protosoë, rickettsias, anaplasmas, virusse, spirochaete en bakterië) geïdentifiseer met die uitsondering van bosluistoksikose waarin patogeniese agente nie betrok= ke is nie. Dus is die veroorsakende agente geassosieerd met bosluisparalise, sweetsiekte en bosluistoksikose veroorsaak deur die Sandtampan nog nie geïdentifiseer nie. Hierdie agente ontwikkel oënskynlik slegs in die invertebraat gasheer (die bosluis) en 'n tydige verwydering van die vertebraat gasheer, word gevolg deur 'n verligting van die simptome. Diere wat herstel ontwikkel 'n langdurige immuniteit.

Die waarnemings betreffende bosluisparalise en sweetsiekte is heelwaarskynlik ook van toepassing op bos=

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luistoksikose veroorsaak deur die Sandtampan.

Daar is aanduidings dat toksiene in die speeksel=agtige sekresie van bosluise teenwoordig is (S 5). In hierdie proefskrif word bewyse gelewer dat dit die geval is; 'n metode vir die isolasie van 'n toksien in die sekresie is beskryf en data word gegee betreffende die chemiese samestelling van die sekresie. Verder is som=mige chemiese komponente en aktiwiteite in die speeksel=agtige sekresie van die bontbosluis Amblyomma hebraeum ondersoek en die resultate verstrek. Sandtampan bosluise gebruik vir die ondersoeke is in die Noord-Wes Kaap Kala=hari versamel met behulp van droë-ys (S 5). Die speeksel=agtige sekresies van die bosluise is verkry deur parasim=patiese stimulasie soos deur Howell (S 6) beskryf. Som=mige eienskappe van die sekresie is in Tabel S 1 aangetoon. Die ensimatiese aktiwiteite bevorder ongetwyfeld die in=dringing van die toksien in die weefsels van die gasheer.

Die toksiese aktiwiteite is geïsoleer deur middel van jel- en ioonuitruilingschromatografie en iso-elektriese fokusering. Sommige eienskappe van die toksiese komponent word in Tabel S 2 aangetoon.

Verwydering van die koolhidraat-gedeelte of S-karbok=simetilering van die toksien lei tot 'n algehele verlies in toksiese aktiwiteit. 'n Nie-toksiese glikopeptied wat die meeste van bostaande karaktertrekke vertoon is ook in die speekselagtige sekresie gevind. Hierdie verbinding het slegs enkele verskille vertoon met betrekking tot die iso-elektriese punt (0,3 eenhede laer) en aminosuurvolgorde (Tabel S 3). 'n Nadere beskouing van die strukturele ver=wantskappe van hierdie twee glikoproteïene kan uiteindelik

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lei tot die oplossing van die probleem betreffende die strukturele vereistes noodsaaklik vir toksisiteit.

Die sindrome veroorsaak deur die speekselagtige sekresie (S 25) wys dat die toksien sy effek uitoefen deur die kapillêre permeabiliteit of breekbaarheid te verhoog. Aangesien 'n metode beskryf is vir die suiwering van die toksien en aangesien sy eienskappe gedeeltelik beskryf is, kan die biochemiese letsel(s) verder ondersoek word.

Die totale vry aminosure teenwoordig in die speek= selagtige sekresie van die bontbosluis Amblyomma hebraeum is bepaal. 'n Totaal van 1553 µg aminosure per ml sekresie is gevind. Hierdie waarde is ongeveer 3 maal hoër as die waarde vir die Sandtampan en ongeveer 20 keer hoër as vir menslike speeksel.

Die sekresie van hierdie bosluis is gefraksioneer deur jelchromatografie. Een van die fraksies het swak hialuronidase aktiwiteit vertoon en is homogeen bevind deur sedimentasie ewewigssentrifugasie. Die molekulêre massa, bepaal deur die laasgenoemde metode is 9680.

Die aminosuursamestelling van hierdie fraksie is met weinige uitsondering soortgelyk aan dié van die hialuroni= dase geïsoleer van bees testikulêre weefsel.

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TABEL S 1 EIENSKAPPE VAN DIE SPEEKSELAGTIGE SEKRESIE VAN *O. savignyi*

Eienskappe van die sekresie		Verwysing na metode aangewend
Opbrengs	48 nl mg^{-1} bosluis liggaamsmassa	S 6
pH	7,9	
Absorpsie maksimum	276 nm	
Totale stikstof	3,6 mg ml^{-1}	S 7
Nie-proteïen stikstof	0,5 mg ml^{-1}	S 8
Totale aminosure	21,2 mg ml^{-1}	S 9
Vry aminosure	0,55 mg ml^{-1}	S 8
Toksisiteit (LD_{50} vir muise)	0,21 ml kg^{-1} liggaamsmassa	S 10
Hittestabiliteit van die toksiese aktiwiteit	stabiel tot 82 °C	
Cholienesterase aktiwiteit	9,7 µmol substraat gehidroliseer min^{-1} ml^{-1}	S 11
Proteolitiese aktiwiteit bepaal met die volgende substrate		
(i) Kaseïen	2,8X10^{-2} Kunitz eenhede	S 12
(ii) Bensoïel-L-arginienetielester	3,3 µmol substraat gehidroliseer min^{-1} ml^{-1}	S 13
(iii) Asetiel-L-tirosienetielester	geen aktiwiteit nie	S 14
(iv) Bensoïel-L-arginien-p-nitroanilied	0,2 µmol substraat gehidroliseer min^{-1} ml^{-1}	S 15
(v) Asetielfenielalanien-L-diiodotirosien	0,05 µmol substraat gehidroliseer min^{-1} ml^{-1}	S 16
Hialuronidase aktiwiteit	71,4 Eenhede ml^{-1}	S 17

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TABEL S 2 EIENSKAPPE VAN DIE TOKSIESE KOMPONENT

Eienskappe van die toksiese komponent		Verwysing na na metode aangewend
Oprbrengs	3-4 mg ml^{-1} sekresies	S 18
Toksisiteit (hoeveelheid onder=huids in 10 g muise ingespuit)	0,4 mg veroorsaak die dood na 90 min	S 18
Absorpsie maksimum	278 nm	
Stikstofinhoud	14,9%	S 7
Iso-elektriese punt	5,1	S 7
Aminosuurinhoud	72,1%	S 9
Totale koolhidraatinhoud	11,3%	S 19
Sialiensuurinhoud	0,9%	S 20
Galaktosamien- en glukosamien inhoud	afwesig	S 21
Koolhidraat-peptiedbinding	O-glikosidiese tipe waarin serine of treonine betrokke is	S 22
Molekulêre massa	6800	S 23
Molekulêre massa van peptied	4652	S 22, S 23
Molekulêre massa van koolhi=draatgedeelte	(6800-4652)=2148	
Ensimatiese aktiwiteite t o v substrate in Tabel S 1 vermeld	geen aktiwiteit	Sien Tabel S 1

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TABEL S 3 GEDEELTELIKE AMINOSUUR VOLGORDE VAN DIE TOKSIESE EN NAVERWANTE NIE-TOKSIESE KOMPONENT, BEPAAL DEUR DIE METODE VAN EDMAN (S 24)

		1 ... 10
Toksiese komponent	*(TK)*	*Gly-CMCys-Pro-Pro-Gly-Val-Pro-Thr-Arg-Ala-*
Nie-toksiese komponent	(NTK)	Asp-CMCys-Pro-Pro-Thr-Lys-Pro-Thr-Arg-Ala-
		11 ... 20
	(TK)	*Tyr—Val-Ala-Phe-Val-Glu-Gly- - -Gly-Ala-*
	(NTK)	Tyr—Val-Ala-Phe- - -Glu-Gly-Glu- - -Ala-
		21 ... 30
	(TK)	*Tyr—Leu-Ile-Val-Val-Thr-Leu- - -Leu- - -*
	(NTK)	Tyr—Leu-Ile-Val-Val-Ser- - -Asp-Leu- - -
		31
	(TK)	*Ala—Leu-*
	(NTK)	Ala-

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