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IMMUNOLOGY OF THE PROTEIN TOXIN OF BACTERIAL INSECT PATHOGENS

by

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Summary

The toxicity for certain insect larvae of the parasporal protein crystal of the bacterium Bacillus thuringiensis is well documented. There is evidence that the crystal is a protoxin which is activated to form a toxin by digestion in the insect gut. It is believed that difference in host-specificity and toxicity of strains of B. thuringiensis may be reflected by differences in the composition of the crystal toxins.

A method was developed for the isolation of pure crystals from mature cultures of B. thuringiensis consisting of 50% spores and 50% crystals. The method is essentially a 2-stage process; in the first stage a flotation technique removes 60% of the spores from the mixture and in the second stage a phase separation technique completes the purification. 60-65% of the crystals were recovered from the culture at a purity of 99%.

Preparations of crystals from 23 strains of B. thuringiensis were used to prepare antisera. A selection from these antisera enabled an antigenic analysis of solutions of the crystals from 94 strains of B. thuringiensis to be carried out using the Ouchterlony gel diffusion technique. Although crystals stimulated production of only one antibody, solutions of many of the crystals contained several antigens. The 94 strains were divisible into 32 groups on the basis of the antigens of crystal solutions. Altogether 11 different crystal antigens were distinguished in the strains of B. thuringiensis investigated.

Crystals from 93 strains of B. thuringiensis were digested by the gut juice of larvae of Pieris brassicae. Ouchterlony gel diffusion indicated that digestion of the crystals resulted in loss of 4 out of the 11 antigens detected in crystal solutions; other antigens were more resistant to digestion. Some antigens not detectable in the crystal solutions appeared in the digests. The antigenic composition of crystal digests was less varied than that of crystal solutions.

The soluble products of digestion of crystals of the Berliner strain of B. thuringiensis were investigated by gel filtration. Two fractions were separated: fraction C1 was composed of material of molecular weight 200,000 and fraction C2 was composed of material of molecular weight 5,000-10,000. Fraction C1 was not toxic on injection into the haemocoel of larvae of P. brassicae although fraction C2 was toxic by this route and so is believed to contain the activated toxin. Digestion experiments also indicated that fraction C1 was the precursor of fraction C2 in the digestion process. 3 antigens were detected in fraction C2 of the crystal digest.

A gel filtration technique was designed to investigate the association of the antigens present in fraction C2 with particular molecules. Gel filtration of mixtures of monospecific antisera and fraction C2 indicated that the 3 antigens in fraction C2 were present as different antigenic sites on one peptide molecule. These mixtures were fed to larvae of P. brassicae. The results indicated that one of the 3 antigens was necessary for the toxic function of fraction C2. Neutralisation of the other 2 antigens individually had no apparent effect on toxicity; neutralisation of both of these antigens together reduced toxicity.

Evidence is presented to suggest that the protoxin crystal is activated by insect gut juices by digestion to yield a single toxic fraction of comparatively low molecular weight.

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INTRODUCTION

The group of strains of aerobic, spore-forming bacteria now collectively referred to as *Bacillus thuringiensis* possesses one important property which makes the group readily distinguishable from all other members of the genus *Bacillus*, *i.e.* the presence in sporing cultures of microscopically-visible crystals.

The pathogenicity of these crystal-formers for certain insect larvae has been shown to be due, in large part, to the presence of these crystals which are toxic on ingestion. While much is known about the applicability of these bacteria to the control of insect pests of agriculture, forestry and stored food products, relatively few fundamental studies of the crystal have been described. It is known that the crystal is protein and exerts a toxic action when enzymatically solubilised in the gut of susceptible insects. Little is known, however, about the nature of the toxic sub-units at a molecular level or about the mode of action of the toxin on the insect. The further problem of variations in host-specificity and pathogenicity among the strains has not yet been related to any feature of the bacteria or to the toxin crystal although variations in crystal toxicity are known.

The aim of the work of this thesis is to investigate the antigenic and toxic determinants of the crystal protoxin and of the toxin sub-units and to estimate the extent of the relationship between these two types of determinants.

The crystal-forming bacteria have been described under a wide variety of names by various authors at different times. Variously, the crystal-formers have been regarded as sub-species of the related organism B. cereus or as varieties of a distinct species B. thuringiensis or as a set of distinct species. For example, the Berliner strain has been referred to under the following names by different authors:-

Bacillus cereus var. thuringiensis Berliner
(Smith, Gordon & Clark - 1952)

Bacillus thuringiensis var. thuringiensis Berliner
(Krywienczyk & Angus - 1960)

Bacillus thuringiensis Berliner
(Hannay & Fitz-James - 1955)

This confusion in nomenclature cannot be avoided without amending the names of the authors who are quoted throughout the thesis. In the writer's opinion such a procedure would involve an unjustifiable exercise of an editor's function and accordingly names of strains as quoted by the original authors are retained.

The following review covers the present field of knowledge of the crystal-forming bacteria with particular reference to the crystal itself and to the work described in this thesis. This work includes methods of crystal preparation, antigenic analysis of crystal extracts, crystal digestion studies and toxicity studies. The literature relevant to these topics and to the final discussion is described.

1. Discovery of Bacillus thuringiensis

In 1902, Ishiwata isolated a spore-forming, aerobic bacterium from dying silkworm larvae (Bombyx mori L.). Ishiwata described his work with the bacterium in 1905 and the organism was named "sotto-Bacillen"; "sotto" means "sudden collapse" in Japanese and refers to the bahaviour of infected silkworms. The existence of parasporal bodies was not mentioned by Ishiwata and it must be assumed that if they were seen at all under the microscope, Ishiwata did not consider them to be significant. Indeed, the work of Ishiwata which demonstrated the pathogenicity of old cultures of Bacillus sotto for Bombyx mori can only be regarded as preliminary to the work of later researchers.

2. Discovery of the parasporal body or crystal

Berliner (1911) examined larvae of the flour or meal moth Anagasta (Ephestia) kühniella Zeller and isolated from diseased larvae a bacterium which he named Bacillus thuringiensis referring to the German province of Thuringia where the work was carried out. Berliner described the isolate as a Gram-positive peritrichously flagellated spore-forming rod. The first published description of the parasporal body must be credited to Berliner who noted that as sporulation of Bacillus thuringiensis on solid medium proceeded, each vegetative cell developed a spore in one half of the cell. At the same time there was a condensation of

material in the other half of the cell instead of a gradual disappearance of cell contents during spore formation as normally occurs in most of the spore-forming bacteria. the condensing material became spherical at first but later enlarged somewhat and developed into an irregular rhomboid body which Berliner called the "Restkörper" or residual body.

Mattes in 1927 isolated B. thuringiensis from A. kühniella and repeated the observations of Berliner on the "Restkörper". He noted that when sporing cells were stained with Giemsa, part of the "sporen anlage" was stained bright red and also part of the cell where the "Restkörper" formed. He suggested that the granules were part of the bacterial nuclear material on the basis of their staining with the nuclear stain Giemsa. Although Berliner and Mattes recognised that the "Restkörper" had a regular rhomboid shape, neither of these workers stated or suggested that the "Restkörper" or parasporal body was crystalline in nature.

After the paper by Mattes (1927), a considerable period elapsed before Hannay (1953) examined B. thuringiensis from the point of view of its sporulation after the report by Smith, Gordon & Clark (1952) that the organism known to them as B. cereus var. thuringiensis had a tendency for its spores to lie obliquely in the cell rather than parallel to the axis of the sporulating vegetative cell as is usual in spore-forming bacteria. Hannay noted that in sporulating cells the spores

were always accompanied by what appeared to be diamond-shaped crystals. This was the first report in the literature of a possible crystalline structure of the parasporal body of B. thuringiensis. Further, although there was considerable variation in size of the crystals, their shape was constant. After sporulation was complete, both spores and crystals were set free and did not undergo any further change.

Since the discovery of the crystalline nature of the parasporal body, there has been a considerable degree of interest aroused in this particle since protein crystals are very rare in nature. Resulting lines of research into the fundamental nature of the crystal are summarised in further sections of this review of the literature.

3. The crystal as a protoxin

Aoki & Chigasaki - (1915a, 1915b) examined the strain Bacillus sotto isolated by Ishiwata and a significant part of this work was the finding that young vegetative cells were incapable of causing disease in silkworm larvae and, in fact, were destroyed in the insect gut. Only older cultures, those that contained spores, were capable of causing disease. This contrasts with most bacterial pathogens where young cultures are generally as virulent as older cultures and was interesting from this point of view. When spores were fed to the larvae, they survived and apparently did not germinate. Aoki & Chigasaki concluded that larvae were killed by the

action of a substance present in the culture. The toxic substance was not present in the medium used for culturing the bacteria and was not filterable through clay candles. Boiling the culture for ten minutes destroyed its toxic activity as did iodine, formaldehyde, mercuric chloride and alcohol.

Much later, Angus - (1956a) reported some work which had been done before the discovery of the crystal by Hannay - (1953). These earlier experiments indicated to Angus that the spore itself and any product directly connected with spore germination could not be responsible for the toxicity of cultures of B. sotto. Culture filtrates and supernatants were not toxic and filtered spore disintegrates were not toxic either although disintegrates of old cultures were toxic. The results therefore implicated a particle other than the spore as the source of a toxin.

Evidence to support the idea of a toxic particle came from Hannay - (1953). He examined a number of spore-forming insect pathogens considered then to be variants of B. cereus according to the classification of Smith, Gordon & Clark - (1952). The majority of this group formed crystals on sporulation and all the strains of B. cereus isolated from sources such as soil and food other than insects and which were not known to be pathogenic for insects did not form crystals. Hannay concluded with the speculation "A

biological phenomenon in which crystalline inclusions have only been found in strains of bacteria pathogenic for insects must inevitably, in the absence of additional observations lead to some speculation as to the possible association of inclusions with pathogenicity".

These conclusions were supported by Steinhaus & Jerrel - (1954) who examined 51 strains of the genus Bacillus by testing them for pathogenicity against larvae of Colias philodice eurytheme Boisd. and Junonia coenia Hbn. Eleven strains of B. thuringiensis which formed crystals were the most active and it was concluded that the crystal was responsible for the toxic action of the bacterial suspensions. Furthermore, Angus - (1954) tested two preparations of spores and crystals obtained by differential contrifugation which varied in the ratio of spres to crystals. Toxicity varied as the crystal count and was independent of the number of spores present.

An imaginative approach to understanding the toxic nature of the crystal came from Angus - (1954) and represented an important step forward. When old cultures of B. sotto were treated with clarified silkworm gut juice an extract was obtained which was toxic when fed to the silkworms. Hannay - (1953) had reported that the crystals were readily soluble in dilute alkali. Angus treated the cultures of B. sotto with alkali and filtered the crystal

solution. When this sterile filtrate was fed to silkworms, paralysis ensued. The results in a later paper are shown below (after Angus - (1956a).

Table 1

Effect on silkworms of feeding various fractions of a digest of whole cultures (spores and crystals) of B. sotto with silkworm gut juice

DIGEST FRACTION	NO. OF LARVAE TESTED	AMOUNT (ml.)	DEATH (D) OR PARALYSIS (P) 4h.	24h.	NO. OF VIABLE SPORES/DOSE
Gut juice (control)	10	0.1	0	0	-
Undigested culture sediment (spores and crystals)	10	0.1	10P	10D	too numerous to count
Digest supernatant fluid	20	0.1	20P	20D	250
Filtered digest supernatant fluid	10	0.1	10P	10D	sterile
	65	0.1	46P	46D	sterile
Heated digest supernatant fluid	10	0.1	0	0	sterile

The idea which inspired Angus to use silkworm gut juice to extract or activate the crystal apparently derives from the work of Bolle which according to Angus - (1956a) showed that the protein inclusion body of silkworm polyhedrosis virus was dissolved by silkworm gut juice. Angus - (1956a) also noted the analogy (previously mentioned by Hannay - (1953) to the work of Bergold who, according to Angus - (1956a), found that viral polyhedral protein which is soluble in silkworm gut juice (pH 9.5-10) is also soluble in alkali.

One point of interest is the lack of toxicity to the silkworm of alkali extracts of the crystals of B. sotto when injected into the haemocoel (bloodstream) and yet the crystals were toxic when administered orally. Most other bacterial toxins are toxic on injection. Angus - (1956b) offered a number of explanations; "the toxin is a protoxin which must be either activated or converted in the gut to an active compound; the sensitive site or receptor site is exposed only in the gut; the toxin is inactivated or inhibited by some component of the blood".

Further work along these lines by Lecadet & Martouret - (1962) investigated the lysis or digestion of B.thuringiensis Berliner crystals in vitro by the chyle of the cabbage butterfly Pieris brassicae. It was thought that the crystal protein was a protoxin which was broken down to release a substance toxic on injection. Lecadet & Martouret - (1962) mixed crystals with chyle at 30°C. in the presence of ethylene diaminotetraacetic acid 10^{-3}M to prevent inhibition of proteases by Ca^{++} and Mg^{++}. The supernatant of the digestion of the crystals was separated into two fractions by dialysis and the table overleaf shows the results of ingestion and injection of these fractions on larvae of P. brassicae.

Table 2

Lethal effect on larvae of Pieris brassicae on ingestion and injection of dialysable and non-dialysable fractions of B. thuringiensis Berliner crystals digested with P. brassicae chyle

FRACTION	N in (µg/ml.)	INGESTION (10 LARVAE) DEATHS AFTER DAYS			INJECTION (10 LARVAE) DEATHS AFTER DAYS		
		3	5	10	3	5	10
non-dialysable	225	7	10	10	10	10	10
	22.5	5	7	10	6	7	8
	2.25	2	8	9	1	3	6
	0.45	3	4	6	6	9	9
dialysable	224	8	10	10	10	10	10
	112	8	10	10	10	10	10
	22.4	8	10	10	10	10	10

Controls of buffer and gut juice were neither toxic by injection nor by ingestion. The larvae were tested in their 4th instar. Deaths were found to correspond to reductions in the amount of food consumed as compared with control groups of larvae. The results showed that both fractions were toxic by both ingestion and injection though whole crystals had been found to be toxic only on ingestion. The non-dialysable fraction had a molecular weight of more than 45,000 as estimated by its exclusion on the gel filtration medium 'Sephadex' G-75 and the dialysable fraction had a molecular weight of a maximum of 12,000-15,000, presumably estimated by its dialysability. No evidence was put forward, however, that the two fractions had been completely separated. Lecadet & Martouret concluded that the same toxic group was carried by

molecules of different sizes produced by a two-stage reaction in which the crystal is dissolved, then enzymatically lysed to give at least two fractions of unequal sizes with the liberation of the toxic group. This latter reaction transformed the protoxin crystal into the true toxin active on injection into the haemocoel or body-cavity of P. brassicae.

However, Benz - (1962) published an account of toxicity experiments in which larvae of P. brassicae were injected with their gut juice. It was found that the larvae suffered complete paralysis when injected with untreated chyle and it was suggested that there was a toxin in the chyle (gut juice) which was responsible for the toxic effect on its injection into the haemocoel. This toxin was precipitated by freezing and thawing. Since Lecadet & Martouret - (1962) had lysed crystals with chyle which was only partially purified, there existed the possibility that some of the toxic effect of the crystal lysates was due to the Benz toxin. Yet Benz found that his toxin was not dialysable so it is unlikely that it was present in the dialysable fraction of the crystal lysate of Lecadet & Martouret who might have frozen the chyle at some stage in the experiment thereby inactivating the Benz toxin although there is no mention of this in their paper.

Next Heimpel - (1963) suggested that Lecadet & Martouret had extracted a toxic material from the gut juice rather than

caused the release of a toxin from the crystals. In reply to this criticism of their work, Lecadet & Martouret - (1965) carried out a series of experiments in which the chyle was frozen at -22°, thawed, centrifuged and the supernatant used for crystal lysis after several purification stages by the method of Lecadet & Dedonder - (1964a) which involves the partial purification by column chromatography of proteases from the chyle capable of lysing the crystals. The chyle was tested at several stages in its purification for its possible toxic effect on P. brassicae by forced ingestion and by intrahaemocoelic injection using the same concentration as would have been present in crystal lysates. It was found that the chyle was non-toxic at all stages. Lecadet & Martouret were satisfied as to the bacterial origin of the toxin.

4. Properties of the crystal

(a) Solubility of the crystal

Hannay - (1953) noted that the crystals of B. thuringiensis Berliner were readily soluble in dilute alkali but were insoluble in water, physiological saline, ethanol, methanol, ether, chloroform, benzene and acetone. The process of dissolution in alkali was followed under the light microscope and the crystals were seen to swell while retaining their shape, then lose their refractility and finally disappear.

This was followed by a closer investigation. Hannay & Fitz-James - (1955) examined material extracted from a mixture of spores and crystals by sodium hydroxide solutions of increasing pH and noted changes in the appearance of the crystals. Chemical analysis and U.V. absorption spectra of alkali extracts of crystals showed that they contained a protein or proteins with an isoelectric point between 4.5 and 5.2. Hannay & Fitz-James suggested that the high pH (12.2) of sodium hydroxide required to dissolve the crystals completely might have split the protein molecules into smaller units. Table 3 below shows that as pH was increased, crystals gradually became swollen, less refractile and began to dissolve. The dissolved crystal proteins were precipitated in varying amounts at acid pHs.

Table 3

The nitrogen and phosphorus content of soluble proteins extracted by alkali from different batches of crystal/spore mixtures of B. thuringiensis Berliner at successive pHs and the morphology of the crystals at these pHs

SUCCESSIVE EXTRACTIONS AT pH	COMPOSITION OF ALKALI-SOLUBILISED MATERIAL AT pH		CRYSTAL MORPHOLOGY
	NITROGEN, % OF DRY WEIGHT	PHOSPHORUS, % OF DRY WEIGHT	
7.0-10.0	12.8-13.6	0.08-0.13	Refractile
-10.7	13.3-13.7	0.08-0.19	Loss of refractility
-11.8	13.3-13.6	0.08-0.19	Swelling to form 'ghosts'.
-12.5	14.0-17.1	0.00-0.04	No crystal structure seen.

At pH 7.0-10.0 the soluble protein extracted by alkali was precipitated by neutralisation; yields were variable. When the pH was raised to 10.0-11.8, small amounts of soluble protein were extracted and could be precipitated at pH 4.5-4.6. Then at pH 11.8-12.5 soluble protein was extracted in large amounts and could be precipitated at pH 4.9-5.2.

Angus - (1956b) found that toxic material could be extracted from sporulated cultures of B. sotto at about pH 10.0 and above. The optimum molarity of sodium hydroxide for extraction was 0.02-0.05 which gave the most toxic extract. Beyond 0.05 N the material was less toxic and Angus concluded that strong alkali inactivated the toxin. He applies this finding in an elaborate method for extracting toxic protein from B. sotto. Although extracts of crystals were more toxic when more dilute alkali was used, Angus found that the most toxic extracts were only 7% as toxic as whole crystals on a weight basis. This was confirmed by Fitz-James, Toumanoff & Young - (1958) who dissolved crystals of B. cereus var. alesti in 0.1 N sodium hydroxide and found that crystals were 2-3 times more toxic than the alkali extracts. The crystal protein did not dissolve until the pH was higher than 11.8 and even then it was seen that about 84% of the crystal protein at pH 12.2 could be centrifuged out at 10,000g. It is not certain

whether the crystals were exposed to alkali for a time sufficient to give complete dissolution.

The results of Fitz-James, Toumanoff & Young - (1958) are summarised below.

Table 4

Effect of pH on morphology and solubility of B. cereus var. alesti crystals

pH OF CRYSTAL SUBTRACTION	CRYSTAL MORPHOLOGY	% NITROGEN EXTRACTED
pH 6.0-10.5	Loss of refractility and slight swelling	0.53
10.5-11.0	Marked swelling	0.65
11.0-11.8	Some disintegration of swollen crystals	-
11.0-12.2	Marked loss of crystal structure	9.0
10.8 wash	No precipitate at pH 4.5	

These results led to attempts at finding less drastic means of dissolving crystals than by high pH. Young & Fitz-James - (1959) showed that the crystal of B. cereus var. alesti, which was soluble in alkali at pH greater than 12, could be dissolved completely at pH 11.5 in the presence of the reducing agent thioglycollate 1% and it was suggested that the existence of disulphide bonds between sulphur-containing amino acids in the protein chains in the crystal could result in a relatively insoluble protein. Lecadet - (1966) investigated dissolution of the crystal of B. thuringiensis var. Berliner in the presence of the reducing agents: thioglycollate, cysteine and mercaptoethanol in the pH range 8-11.5. Dissolution of the crystals was

proportional to the concentration of reducing agent and was prevented by adding cystine which allows reformation of disulphide bonds. However, there is no evidence that the fragments released from the crystal as described by Lecadet correspond to those released by enzymic lysis in the insect gut.

(b) Chemical composition of the crystal

A number of papers have reported that the crystal is composed largely or wholly of protein. Hannay & Fitz-James - (1955) found that solubilised crystals of the Berliner strain had a U. V. absorption spectrum typical of a protein. Nitrogen content of 14-17% was also typical of a protein. Phosphorus content was negligible (0.00-0.04%) and must be construed as contamination. The amino acids of an acid hydrolysate of the crystals were chromatographed; 17 amino acids were present. Angus - (1956b) showed that the crystals contained 18% nitrogen and 0.05% phosphorus and investigated the amino acid composition of both whole crystals and the extracted protein more thoroughly than Hannay & Fitz-James - (1955). The amino acid composition of whole crystals and the extracted protein are very similar.

Lecadet - (1965) extended analysis of the crystals of both Berliner and Anduze strains. Sugars were absent by the orcinol method. Lecadet noted a high proportion (25%) of

the dicarboxylic amino acids aspartic and glutamic acids which explains the acidic nature of the crystal protein.

(c) Microscopy

Hannay & Fitz-James - (1955) examined crystals of B. thuringiensis Berliner by light and electron microscopes and concluded that the crystals were very regular in shape and that they were tetragonal as judged by their shadow-casts in electron micrographs. Crystal sections were prepared and, not surprisingly, were four-sided. Shadowing revealed that the edges of the crystals were serrated and these serrations corresponded to parallel rows of material extending round the edges. In some cases it was noted that the rows were composed of spherical units which were also found in alkali-dispersed crystals but no attempt was made to estimate the size or unit arrangement of these spheres. Manipulation of the electron beam of the electron microscope was used to produce "shells" composed of the outer layers of crystals. This type of technique requires a careful interpretation of the results and so it was thought that the surface pattern noted by Hannay & Fitz-James - (1955) could have been an artefact. The work of Dawson, Norris & Watson - (1958) made use of replica techniques in an attempt to avoid crystal distortion and concluded that the ridging was a feature of the crystal surface and had a periodicity of 290Å. Thin sections showed a 40Å periodic line structure.

Norris & Watson - (1960) in a further paper concluded that the ridging reflected molecular packing in the crystal rather than some form of surface membrane still adhering to the crystals after rupture of the cell.

Further studies include those of Labaw - (1961, 1964) who described the crystals as composed of tetramolecular face-centered-cubic unit cells 123Å on an edge. The protein molecules were 87Å in diameter. The work of Holmes & Monro - (1965) estimated the molecular weight of the spherical units as 230,000, but Vanková & Králík - (1966) found that the crystals were composed of globules whose average size varied in a number of different strains of B. thuringiensis; thus the molecular weight of the protoxin may vary from one strain to another.

Electron microscopy has provided good evidence of the crystalline structure of the parasporal body thereby confirming the opinion of the earlier light microscope studies of Hannay & Fitz-James - (1955) where the regular sub-structure of the crystal was not visible.

Although electron microscopy has resulted in more detailed concepts of the crystal sub-structure, it has added little to our understanding of the mechanism by which the crystal protein is crystallised in the bacterial cell and subsequently digested in the gut of the insect. It is possible that future techniques aided by more detailed

analysis of the crystal sub-units may enable us to understand this still mysterious process.

5. Action of spores and crystals of B. thuringiensis on insect larvae

The toxicity to silkworms of the crystal protein of B. thuringiensis has been well established (Angus - 1954, 1956a; Bonnefoi & Beguin - 1959; Fitz-James, Toumanoff & Young - 1958; Steinhaus & Jerrel - 1954) and an average LD50 of 0.5µgm. of toxin per gram of silkworm was obtained with B. sotto toxin (Angus - 1956b).

Angus - (1956a) and Angus & Heimpel - (1956) described the effects of feeding silkworms with leaves which had been coated with spores and crystals of B. sotto and noted a series of symptoms. These were, firstly, a cessation of feeding and sluggishness of movement followed by a slight distension of the body wall in the abdominal region. Larvae stopped feeding after 30 minutes and did not feed again. The larvae were then motionless and they lost the ability to grasp with their legs. Paralysis then set in and since it was irreversible, death followed invariably. Various other appearances have been reported, e.g. sluggishness, regurgitation, diarrhoea and cessation of feeding (Heimpel & Angus - 1959).

Angus & Heimpel - (1959) extended tests on silkworms to three species of forest larvae tested with spores and

crystals of B. entomocidus var. entomocidus applied on foliage and fed to the larvae. Most larvae died 48h. after consuming contaminated foliage. The most noticeable effect was a reduction in the amount of foodplant consumed by treated larvae which was taken as indicating reluctance or inability of the larvae to feed. These larvae did not resume feeding when placed on clean foliage. Infected larvae produced very much less frass than normal larvae and Angus & Heimpel considered this to be due to gut paralysis. The species of larvae investigated did not show general paralysis as did Bombyx mori.

In a critical examination of the effects of B. sotto toxin on silkworms, Angus & Heimpel - (1956) reported that when larvae were fed either extracted toxin or spores and crystals, the pH of their blood increased by 1.5 units with a corresponding decrease in the alkalinity of the midgut and these pH changes appeared to correspond with onset of paralysis. This relationship was further established when injection of normal larvae with buffers giving a bloodstream pH of 8.1 resulted in a similar paralysis.

Further, Angus & Heimpel - (1959) showed that other species of susceptible larvae which did not show complete paralysis after having been fed spores and crystals showed no changes in bloodstream pH. Since the difference between gut and bloodstream pH was similar in larvae which showed

complete paralysis and in those that did not, it was concluded that there were two distinct types of response. This work was more fully investigated by Heimpel & Angus - (1959), who used X-ray and histological studies. They divided susceptible larvae into three groups on the basis of their behaviour after ingesting spores and crystals of the Berliner strain. Both type I and II suffered rapid onset of mid-gut paralysis but type I was the only type of the three to suffer general paralysis and this was due to an increase in blood pH possibly caused by leakage of gut contents through a damaged gut which did not occur in type II; type III was represented solely by Anagasta kühniella which was not affected by extracted toxin alone nor by spores alone and both spores and crystals were required to give a lethal effect.

Although the results indicated a difference between types I and II, Heimpel & Angus - (1959) rightly pointed out that it would be presumptious to suggest that this distinction reflects differences in the mode of action and, in fact, intermediates between types I and II have been reported. It remains to be seen whether this distinction between types I and II will be modified as the result of further experiments.

Histopathological studies in the same paper suggested that ingestion of the toxin was followed by a breakdown of

the cells lining the mid-gut. Sections showed that the cells forming the continuous-lining epithelium had become separated from themselves, and from the basement membrane, and it was tentatively suggested that the toxin affected the mucopolysaccharides which are the cell-cementing substances.

Confirmation of this body of evidence was produced by Fast & Angus - (1965) who studied the rate of transfer of the radio-actively labelled NaC^{14}-carbonate and universally labelled C^{14}-glucose under the influence of spore-free crystals of the sotto strain. A breakdown of the mechanisms which govern selective permeability in the gut-wall had occurred. However, glucose transport was inhibited, while anions were more rapidly transferred as estimated by activity, which suggested that some form of barrier remained to prevent free diffusion across the gut-wall. Thus it appeared that the basement membrane and surrounding connective tissues which according to Spector & Willoughby - (1963) do exhibit some selective permeability, were not affected by the toxin.

Fast & Angus - (1965) and Heimpel & Angus - (1960) proposed, therefore, that the toxin does not affect the cell-cementing substances but affects instead a more specific site such as the actual active transport mechanism.

6. Serological studies of the crystals

There are two papers which are concerned with antigens of crystals of B. thuringiensis varieties (Krywienczyk & Angus - (1960); De Barjac & Lecadet - (1961) and both are strikingly similar. The earlier paper compared the crystals of three strains:- B. thuringiensis var. thuringiensis Berliner, B. thuringiensis var. sotto and B. entomocidus var. entomocidus by preparing antisera to whole crystals and analysing antigen-antibody reactions by gel diffusion using crystal solutions. It was concluded that the crystals were different in at least one antigenic component but at least one antigen was present in all three crystal solutions. Further, it was concluded from toxicity tests on crystal solutions absorbed by antisera to other crystals that absorbed material was non-toxic and therefore all three organisms produced the same toxic protein . Krywienczyk & Angus speculated that the toxin (rather than the protoxin) produced by enzymic lysis in the larval gut was the same in the varieties studied.

De Barjac & Lecadet - (1961) investigated the crystals of the strains Anduze, Berliner and subtoxicus by preparing antisera to both whole crystals and solutions of crystals. It was found that the number of precipitin lines in gel diffusion obtained using antisera to whole crystals did not correspond exactly with those obtained using antisera to

crystal solutions. One antigen was common to all three strains and another antigen common to only Berliner and subtoxicus. The authors did not draw any conclusions from these observations and no suggestion was made as to the function of the various antigens in the crystal.

Much of this thesis is concerned with a detailed antigenic analysis of the crystals from over 90 isolates of B. thuringiensis (Pendleton & Morrison - (1966a).

7. Separation of the spores and crystals

Sporulating cultures of B. thuringiensis varieties contain both spores and crystals in equal numbers. Techniques for the preparation of crystals free from spores and for the preparation of spores free from crystals have arisen from a need to investigate the individual action of spores and crystals in the infectious process and also from an analytical point of view in chemical, biochemical or serological studies. Most of the methods reported in the literature are concerned with preparing spore-free crystals.

Hannay & Fitz-James - (1955) found that chemical analysis of crystal material extracted by alkali from a mixture of spores and crystals did not correspond to a crystal preparation free from spores. Attempts at separating spores and crystals by differential centrifugation

were not successful. Finally two similar methods were used to obtain almost pure crystals. In one method a mixture of spores and crystals was shaken in a Mickle disintegrator so that the spores were disrupted and then the small fragments of spore debris were separated by differential centrifugation from the crystals. In the other method, which was apparently not reliable, spore/crystal suspensions were harvested from their growth medium and autolysed for 8-12 days in the refrigerator. During this time the spores germinated spontaneously and the resulting vegetative cells disintegrated in the absence of nutrients. The crystals which remained unaltered were washed free from debris by differential centrifugation.

Robertson & Heimpel - (1962) published an elaborate and time-consuming method for obtaining crystals from a commercially produced concentrate of B. thuringiensis var. sotto. The purity of the crystal suspension was 98%, with 2% spores. Briefly, the method is based on a number of stages of differential centrifugation and settling followed by washing the centrifuged pellet and swirling the centrifuge tube by hand to obtain crystals from the pellet. The method as described is apparently only applicable to commercial concentrates of one strain (the sotto strain) and is not mentioned further in the literature.

Two papers (Angus - 1959; Bateson - 1965) describe the

separation of crystals by modifying a method which had been used successfully for the purification of insect virus inclusion bodies by separation from host cell remains (Bergold - 1959) using a fluorocarbon $CF_2Cl-CCl_2F$.

The method of Angus - (1959) involved the use of a washed spore/crystal suspension at a concentration of 1.5 gm. of wet cells in 900ml. of water. This was mixed with the above-mentioned fluorocarbon (100ml.) to form an emulsion. On standing, the emulsion separated into two layers - the upper layer was aqueous and contained mostly crystals with a few spores; the lower layer contained a few crystals and mostly spores. The upper layer was treated a further three times with trifluorotrichloroethane to give a crystal suspension of 95% purity. The remaining spores were removed to give a suspension containing more than 99% pure crystals and less than 1% spores by spore germination in nutrient broth followed by dialysis and a final treatment with fluorocarbon.

The method of Bateson - (1965) represented a modification of the method of Angus - (1959) in an attempt to reduce the number of fluorocarbon treatments to two and the time involved to 6h. whilst maintaining crystal purity above 99%. The method also dispensed with the preliminary partial germination of spores in Angus' method.

The sole method for the preparation of spores free from

crystals is the method of Angus - (1956b) where a mixture of spores and crystals was suspended in alkali to dissolve the crystals and the spores removed by centrifugation and freed from crystal protein by repeated washing. It was not determined whether the alkali had any detrimental effect on the spores.

A method of obtaining either free spores or free crystals will be described in this thesis (Pendleton & Morrison - (1966b).

8. Host-specificity and pathogenicity of strains

Heimpel - (1963) reported that approximately 110 Lepidopterous and 8 Dipterous insects comprised the total number of susceptible species then known but indicated that the list was far from complete. At that time there were 27 separate isolates of crystal-forming bacteria which had all been isolated from Lepidoptera.

However, within this group of crystal-forming bacteria it has been noted that there is considerable variation in virulence for susceptible insects and further there is a degree of host-specificity. That is, certain isolates tend to be associated in disease with certain insects (Norris & Burges - 1965).

An early study (Toumanoff & Vago - 1952) showed, although only a few strains of B. thuringiensis were known

at that time, that strains pathogenic for certain insects were scarcely, or not, pathogenic for related species of insects. They noted that amongst the four crystal-forming strains investigated, only one, alesti, was notably virulent for Bombyx mori larvae and related this to the original isolation of alesti from Bombyx mori in the "Flacheries" epizootic (Toumanoff & Vago - 1951).

Further studies include that of Burgerjon & Grison - (1959) who investigated the relative sensitivities of 24 species of Lepidoptera representing ten families, to the strain Anduze. They emphasized that only an approximate qualitative estimation of sensivity had been made since they did not determine the lethal dose or LD_{50}. The relatively high susceptibility of Pieris brassicae was noted. In their paper, Burgerjon & Grison cite a mimeographed document which was circulated by Steinhaus to a limited number of specialists in 1957 and regard their studies as supplementing those reported by Steinhaus.

Toumanoff & Durand - (1961) approached the problem by taking one species of insect Bombyx mori and testing a number of isolates against it taking into consideration the original host of the isolate. For example, the isolate from Plodia interpunctella made by Weiser in Czechoslovakia is pathogenic for silkworms in high doses only, but the isolate of Schvetsova from Galleria mellonella is highly

pathogenic for silkworms. This work, therefore, suggested that there was a variation in host-range within the group of crystal-formers.

The large number of isolates of crystal-formers and the wide range of susceptible insects has deterred a complete study of the host-specificity and virulence. In fact, the work reported in the literature has only investigated a small area of the possible field. Furthermore, no speculations have been made as to the cause or causes of the variation within this group of bacteria. There have been no studies of the toxicity of the crystals in the absence of viable spores from various strains.

9. Classification and taxonomy of the crystal-forming insect pathogens

Before the discovery of the crystal, the close relationship between these bacteria and the common saprophyte *Bacillus cereus* was noted (Toumanoff & Vago - 1952; Smith, Gordon & Clark - 1952). The discovery of the crystal and its association with pathogenicity for insects enabled this characteristic to be used in distinguishing this group from the non-pathogenic *B. cereus*. Thus *B. sotto*, *B. cereus* var. *alesti*, *B. thuringiensis* were recognised as being more closely related to each other than their names suggested. Heimpel & Angus - (1958) compared the biochemical and morphological characteristics of seven

crystal-formers and devised an identification key. The realisation that the group contained some very similar strains was reflected in the suggestion that the strain B. thuringiensis Berliner should become the type species; according to priority this nomenclature should be used: B. thuringiensis var. sotto and B. thuringiensis var. alesti. However, the differences in biochemical reactions and toxicity of some other isolates from lepidopterous larvae caused Heimpel & Angus to form the separate species B. finitimus and B. entomocidus with its variety B. entomocidus var. subtoxicus.

However, Toumanoff & LeCoroller - (1959) suggested that the presence of the crystal was not sufficient to separate the crystal-formers from B. cereus. They divided B. cereus into "acrystallophorous" and "crystallophorous" groups. The crystallophorous group was divided into B. cereus varieties on the basis of action on Loeffler's medium, coagulated serum and egg-yolk agar.

The controversy between Toumanoff & LeCoroller and Heimpel & Angus was simply about whether the crystal-forming bacteria should be considered as varieties of B. cereus or of B. thuringiensis. Heimpel & Angus - (1958) suggested that B. thuringiensis var. thuringiensis be retained as the type species of the crystal-formers for practical purposes, and in 1960 they stated "Most bacteriologists would agree that

bacteriological nomenclature is but a system for the practical differentiation of strains of bacteria. Anyone who takes an academic stand, in defence of the species concept, is on uncertain ground".

In an exhaustive analysis of twenty-four strains of crystal-formers (which were all considered as varieties or biotypes of B. thuringiensis) De Barjac & Bonnefoi - (1962) examined thirty-five cultural and biochemical characters of which ten were selected for classification purposes. The strains were divided into six biochemical groups which corresponded to six serological groups determined on the basis of the H antigen in an agglutination method. Thus both systems appeared valid on the basis of their close fit. Table 5 below shows the system of De Barjac & Bonnefoi - (1962).

Table 5

Classification of 24 strains of crystal-formers by biochemical reactions and flagellar antigens

BIOCHEMICAL GROUP	H ANTIGEN	BIOTYPE	COMMON NAME
I	H_1		berliner
II	H_2		finitimus
III	H_3		alesti
IV	H_4	(a	sotto
		(b	dendrolimus
V	H_5		galleriae
VI	H_6	(a	subtoxicus
		(b	entomocidus

In Table 5 sotto and dendrolimus were distinguished as were subtoxicus and entomocidus.

A further paper by Bonnefoi & De Barjac - (1963) confirmed the six groups delineated in the work of De Barjac & Bonnefoi - (1962) after twenty-six additional strains of B. thuringiensis had been examined. Two new H antigen serotypes were described - serotype 7 (aizawai) and serotype 8 (morrisoni). Also a change was made to serotype 4. A close examination of strains with H antigen IV showed that sub-division of this group on the basis of additional flagellar antigens was possible and the following classification was proposed.

Table 6

Classification of 50 strains of B. thuringiensis by H antigens

SEROTYPE		GROUP
1		berliner
2		finitimus
3		alesti
4	(4a,4b	sotto, dendrolimus
	(4a,4c	kenyae (P.I.L.94)
5		galleriae
6		subtoxicus, entomocidus
7		aizawai
8		morrisoni

The possibility of a third system of classification of B. thuringiensis other than by biochemical reactions and H antigens arose from the work of Norris & Burges - (1963).

It was found that some isolates could be distinguished on the basis of starch-gel electrophoretic analysis of esterases of vegetative cell disintegrates. This work was expanded by Norris - (1964) in an investigation of biochemical reactions, flagellar (H) antigens and esterase analysis of forty-six isolates of B. thuringiensis. The results showed a close correlation between the classifications based on H antigens and esterase analysis. A further serotype was added to the eight of Bonnefoi & De Barjac. The groups distinguished by Norris are described below in Table 7 which duplicates in part the work of De Barjac & Bonnefoi - (1962) and Bonnefoi & De Barjac - (1963).

Table 7

A comparison of the biochemical, serological and esterase characteristics of crystal-forming bacteria

ACETYLMETHYL-CARBINOL PRODUCTION	LECITHINASE	ACID FROM SALICIN	ACID FROM SUCROSE	HYDROLYSIS OF STARCH	H ANTIGEN	SEROTYPE	ESTERASE PATTERN
+	+	+	+	+	I	1	Berliner
+	+	+	+	-	II	2	Finitimus
+	+	-	-	+	III	3	Alesti
+	+	-	+	+	IV(a)	4A	Sotto
+	+	-	-	+	IV(a)	4A	Dendrolimus
+	+	+	-	+	IV(b)	4B	Kenya
+	W	+	-	+	V	5	Galleriae
-	-	-	+	+	VI	6	Entomocidus
+	-	-	+	+	VI	6	Entomocidus
+	+	+	-	+	VII	7	Galleriae
+	-	-	+	+	VIII	8	Morrison
+	+	+	-	+	IX	9	Tolworth

In Table 7 W = reaction weak or variable.

The group aizawai (serotype 7) of Bonnefoi & De Barjac - (1963) became galleriae in the system of Norris - (1964) presumably on the basis of the close similarity between the esterase patterns of serotype 5 (galleriae) and serotype 7.

The group of bacteria named Bacillus thuringiensis has thus been classified on the basis of three separate types of test, biochemical reactions, flagellar antigens and esterase patterns and a good degree of correlation found between these classifications.

In a section of this thesis the results of applying crystal antigenic analysis to classification will be dealt with in detail.

MATERIALS AND METHODS

1. Isolates of Bacillus thuringiensis

The cultures of crystal-forming bacteria which form the basis of the work of this thesis were from a collection gathered by Norris - (1964). Table 8 below lists some of the ninety-four isolates under investigation, the isolating workers, the country of origin, the insect host, and the date of isolation. Stock cultures were maintained by periodic sub-culture on 'Lab-Lemco' agar.

Table 8

Sources of 46 of the 94 isolates of crystal-forming insect pathogens investigated in this thesis

ISOLATE	ISOLATORS	PLACE OF ORIGIN	INSECT HOST	DATE OF ISOLATION
AC 58	Norris	England	Hofmannophila pseudospretella	1963
Bakthane	Ayerst	England	Isolated from 'Bakthane'	1961
berliner	Berliner	Germany	Anagasta kühniella	1911
Brunel	Norris	England	Anagasta	1963
El	Norris	Scotland	Anagasta	1958
galleria allemand	Krieg	Germany	Galleria mellonella	-
Steinhaus 1715	Steinhaus	England	Plodia	1963
thuringiensis LD	Grigarova	Bulgaria	Lymantria	-
thuringiensis LDI	Grigarova	Bulgaria	Lymantria dispar	-
thuringiensis Mattés	Mattés	Germany	Anagasta kühniella	1927
finitimus	MacNamee	Canada	Malacosoma disstria	1956
anduze	Vago	France	Bombyx mori litter	1952
alesti	Toumanoff & Vago	France	Bombyx mori	1951
galleria euxoae	Krieg	Germany	Euxoa segetum	1956
77 MD 0559	-	Germany	Hyponomeuta	1958
77 MD 0658	Béguin	France	Pieris rapae	1959
T 84-A	Aizawa	Japan	-	-
dendrolimus	Talalaev	U.S.S.R.	Dendrolimus sibericus	1956

ISOLATE	ISOLATORS	PLACE OF ORIGIN	INSECT HOST	DATE OF ISOLATION
ACC 1716	Steinhaus	Nigeria	Busseola fusca	1961
ACC 1731	Steinhaus	-	-	1961
Ashman	Norris	-	-	1963
Cardiff Rd.	Norris	-	-	1963
Chicory	Norris	-	-	1963
Eldoret	Norris	Kenya	-	1963
Nairobi	Norris	Kenya	Cadra	1963
PIL 94	Ayerst	England	Trichophaga tapetzella	1961
Rhodesia	Norris	Rhodesia	Cadra	1963
Steinhaus 1748	Steinhaus	U.S.A.	-	1961
Beira	Norris	Mozambique	Plodia	1963
G 1	Norris	Scotland	Galleria	1959
galleriae russe	Schvetsova	Russia	Galleria mellonella	1957
HA-3	Aizawa	Japan	-	-
PIL 106	Norris	England	Plodia interpunctella	-
Pretoria	Norris	S.Africa	-	1963
Schvetsova	Schvetsova	-	-	1957
T 63L4	Aizawa	Japan	-	-
1 H-A	Aizawa	Japan	-	-
ACC 1891	Steinhaus & Marsh	U.S.A.	Paramyelois transitella	1962
entomocidus	Steinhaus	U.S.A.	Aphomia gularis	-
Limassol	Norris	Cyprus	Cadra	1963
subtoxicus	Steinhaus	U.S.A.	Plodia interpunctella	1945
G 2	Norris	Scotland	Galleria	1959
Morrison	Norris	Scotland	Anagasta	1963
Tolworth	Norris	England	Plodia	1963
Kilmarnock	Norris	-	-	1964
Slough	Norris	-	-	1964

2. Media

Dehydrated Oxoid 'Lab-Lemco' agar was dissolved at a concentration of 23g./litre of saline. The medium was sterilised by autoclaving at 10 lb./sq.in. for 20 minutes. Roux flasks were poured on both sides with

approximately 160 ml. of 'Lab-Lemco' agar per side. After overnight incubation at 30°C to check their sterility, each flask was inoculated with the growth from one 'Lab-Lemco' agar slope culture suspended in 5 ml. of saline. The flasks were incubated at 30°C for at least 10 days to obtain completely sporulated cultures and in some cases for 12 days for complete lysis of vegetative cells. Cultures for the preparation of vegetative cells for esterase analysis were incubated at 30°C for 12-16 hours.

3. Microscopy and Staining

A simplified version of Smirnoff's - (1962) method was used for the microscopic examination of cultures for the presence of free crystals and spores. Fixed smears were stained with napthalene black solution at a concentration of 1.5g. in 100ml. of 5% acetic acid for one minute, washed and stained with dilute carbol fuchsin for 30 seconds. The slides were then washed, dried and examined under the oil immersion lens at X1200 magnification. Crystals were stained purple-black, spores were stained red in outline, vegetative cells were stained red-purple throughout and vegetative cell debris was stained pink.

Examination of vegetative cells and vegetative cell disintegrates was done by Gram stain. The degree of disintegration was estimated from the conversion of the intact Gram-positively stained vegetative cells to Gram-negatively

stained disrupted cells.

4. Preparation of Vegetative Cell Disintegrates

Cultures of B. thuringiensis were grown on 'Lab-Lemco' agar in Roux flasks at 30°C for 12-16 hours; the growth was collected in sterile saline and centrifuged at 4,000 rev./min. for 10 minutes. The pellet of vegetative cells was transferred to the glass container of the M.S.E. Mullard 60W ultrasonic disintegrator. Cooling was effected by immersing the container and probe in a beaker of ice/salt mixture. Without this precaution much of the esterase activity of the disintegrate was lost. Disintegration was complete after 5-10 minutes exposure to ultrasound as estimated by the extent of the loss of Gram-positivity of the mixture.

Disintegration in the Braun disintegrator with Ballotini beads required a similar time exposure to vibration but since the volume of cells to be disintegrated was small, it was more convenient to use the ultrasonic disintegrator which also dispensed with the need for Ballotini beads.

Disintegrates were clarified by centrifugation at 4,000 r.p.m. for 15 minutes and stored at -20°C.

5. Preparation of Crystals

Completely sporulated cultures of B. thuringiensis contained equal numbers of spores and crystals with a small

and variable amount of vegetative cell debris. In all strains, except the finitimus strain, the crystals were unattached to the spores. The most useful method of preparing crystals would allow complete separation of spores and crystals so that the crystal purity would be 100% and all the crystals present in the original mixture would be recovered, i.e. at a yield of 100%. In practice this was not achieved; although the purity of 98-99% obtained by the methods described in this section was adequate for our purposes. The maximum yield of crystals was 65% of those present in the original mixture.

Crystals were prepared by several similar techniques based on phase separation with an organic solvent with or without a preliminary flotation technique. These methods are detailed in the order in which they were developed during the work. The purities and yields of crystals obtained by these methods are given in the Results.

(a) Separation without flotation

The method described by Bateson - (1965) was modified to simplify the procedure. Completely sporulated cultures were obtained as described in Section 2. Growth from Roux flasks was collected and washed three times in sterile distilled water and once in sterile saline. The spore/crystal mixture was re-suspended in water at a concentration of 0.05g. wet weight/ml. and filtered through

muslin to remove lumps of agar. To 35 ml. of this suspension were added 30 ml. of 1% sodium sulphate solution and 35 ml. of trifluorotrichloroethane (Arcton 112 manufactured by I.C.I. Ltd.)as organic phase and the whole mixed for 2½ minutes in an M.S.E. homogeniser (Measuring and Scientific Equipment Ltd., 25-28 Buckingham Gate, London, S.W.1.) at about 7,000 r.p.m. The resulting emulsion was allowed to stand for about 15 minutes until the aqueous and organic phases had separated. The aqueous phase was collected by siphon and microscopic examination showed that it contained 96% crystals and 4% spores. The organic phase was discarded. The aqueous layer was centrifuged and the pellet containing 96% crystals and 4% spores was taken up in a small volume of water and mixed with trifluorotrichloroethane as before. The resulting aqueous phase contained 98% crystals and 2% spores. Further treatment with trifluorotrichloroethane did not result in purer crystal preparations.

(b) Separation with flotation

The previous method required two phase separation treatments with trifluorotrichloroethane. Later it was found that it was possible to obtain higher yields of 98% pure crystals after only one phase separation treatment when a preliminary removal by a flotation technique of about 50% of the spores present in the original 50:50 suspension

was carried out. Furthermore, the use of trifluorotrichloroethane was discontinued since carbon tetrachloride was more readily available and gave similar yields and purities of crystals.

The growth was collected and washed as in method (a) but was re-suspended to a concentration of 0.07g. wet weight/ml. instead of 0.05g. wet weight/ml. as in method (a). A convenient volume of this suspension consisting of a 50:50 mixture of spores and crystals was shaken by hand for 5-15 seconds. On standing, a thick scum or froth appeared on the surface of the suspension. Microscopic examination of this froth showed that it consisted of about 95% spores and 5% crystals. The shaken suspension was passed through Whatman's No.1 filter paper when the froth of spores was retained or the froth could be separated by siphon or in a separating funnel. The shaking and removal of froth was repeated a number of times so that the ratio of crystals to spores in the suspension increased from 50:50 to about 80:20 as estimated by microscopic examination. The amount of froth which appeared on the surface became less and less after each shaking.

35 ml. of the suspension containing 80% crystals and 20% spores were mixed with 30 ml. of 1% sodium sulphate and 35 ml. of carbon tetrachloride as in method (a). Crystals were collected as in method (a) after phase separation at a

purity of 98-99%.

(c) Method for obtaining Dendrolimus crystals

It was not possible to obtain crystals from the Dendrolimus strain by method (a) or (b) and the following method was used to obtain 96% pure crystals. No froth appeared on the surface of the shaken 50:50 suspension. 35 ml. of the suspension at a concentration of 0.05g. wet weight/ml. was mixed with 30 ml. of 1% sodium sulphate and 35 ml. of carbon tetrachloride as before. After mixing, the emulsion was allowed to stand overnight when the aqueous layer was collected by siphon and was found to contain 96% crystals and 4% spores.

(d) Separation with solvents other than carbon tetrachloride

A comparison of other organic solvents with carbon tetrachloride in phase separation revealed that substantially different purities and yields of crystals could be obtained. The purities and yields of crystals from the Berliner strain obtained with a variety of solvents are detailed in the Results. From these results it was decided that xylene had several advantages over carbon tetrachloride or trifluorotrichloroethane. In this method (d), xylene was used in place of carbon tetrachloride as in method (b) which was followed to the stage of spore removel by flotation. The residue was homogenised and the emulsion centrifuged at

4,000 r.p.m. for 7-15 minutes which resulted in separation of the emulsion into several layers. The upper layer consisted of xylene, the second layer consisted of a thick paste-like suspension of spores with some crystals in xylene, the third layer was aqueous and contained neither spores nor crystals and the bottom layer was a pellet of crystals at the base of the centrifuge tube. The crystals contained less than 1% spores and were obtained at a yield of 60-65% of those present in the original suspension.

In the previous methods (a), (b), (c) and (d), the washed spore/crystal mixtures contained a variable amount of vegetative cell debris which was present in small amounts in the final crystal preparations. Much of this contamination could be removed by washing the crystals in phosphate buffer M/100 at pH 8.

6. Extraction of Soluble Material from Crystals

Purified crystals or washed mixtures of spores and crystals were suspended in 0.05 N sodium hydroxide at room temperature until dissolution of the crystals was complete as ascertained by microscopic examination. This generally required about one hour. Solutions of crystals intended for immunodiffusion tests were then centrifuged to remove spores and the pH of the supernatant was adjusted to 4.4 with 0.2 M acetate buffer pH 4.4 to precipitate the crystal protein. The precipitate was collected by centrifugation at 4,000 r.p.m.

for 10 minutes and was re-dissolved in 0.05 N sodium hydroxide at a concentration of 5-10 mg. protein/ml. Crystal solutions intended for immunodiffusion tests were maintained at 3°C with thiomersalate as preservative at a concentration of 1/5000.

Extracts of crystals intended for digestion studies were prepared by suspending spore/crystal mixtures in 0.03 N sodium hydroxide rather than 0.05 N sodium hydroxide to prevent undue loss of toxicity (Angus - (1956b). When dissolution of the crystals was complete, the solution was centrifuged to remove spores and the crystal protein was precipitated by adjusting the pH to 4.4 with acetate buffer. The precipitate was washed twice in sterile distilled water and stored at -20°C.

In the case of the finitimus strain where the crystal is firmly attached to the spore, it was not possible to separate the crystals from the spores by phase separation. Instead, preparations of crystal protein for immunisation were prepared by dissolving the crystals attached to the spores in 0.05 N sodium hydroxide. The spores were separated from the crystal protein solution by centrifugation and the supernatant was clarified by passage through a sintered glass filter grade 5/3. Finally, the pH of the solution was adjusted to 4.4 with 0.2 M acetate buffer pH 4.4 when the crystal protein was precipitated.

The precipitate was collected by centrifugation, washed twice in sterile distilled water and re-suspended in water at a concentration of 2 mg./ml. to give an antigen preparation for immunisation.

7. Preparation of Antisera

Rabbits were injected intravenously with 1 ml. of an aqueous suspension of crystals (or a solution of crystal protein in the case of the finitimus isolate) at a concentration of 1.5-2.5 mg. protein/ml. twice per week for three weeks. The rabbits were bled one week after the last injection and the serum was stored at -20°C. Further antiserum was prepared by a single intravenous injection, ten days after the previous bleeding, of 1 ml. of the same crystal suspension and the rabbits were bled one week after the injection.

A complete list of the isolates of B. thuringiensis used for the preparation of antisera to their crystals is given in the Results.

The use of a solution of finitimus crystal protein as opposed to undissolved crystal suspensions with other strains was justified by findings to be discussed later.

8. Digestion of Crystals and Crystal Extracts

Crystals or crystal extract were digested in vitro by crude digestive juice preparations of larvae of the cabbage

butterfly Pieris brassicae. Larvae of this insect at all growth stages readily exuded a droplet of digestive juice at their mouthparts on provocation, but collection of the juice was most convenient during the final instar (growth stage) of the larvae particularly in the first half of this stage when the juice was produced in greatest amount. To collect the juice, the larva was held over the rim of a 4" x ½" tube and its underside compressed gently against the rim in a stroking motion. After collection, the juice was frozen at -20°C, thawed and then centrifuged to remove the Benz toxin which is precipitated by freezing (Benz - 1962). The supernatant which was not purified further was used in digestion studies. A similar procedure was used to obtain digestive juice from the Eri silk-moth Philosamia cynthia.

Crystal preparations or crystal extracts were digested in 0.2 M carbonate/bicarbonate buffer pH 9.5 in the presence of 2×10^{-3}M ethylene diamine-tetra-acetic acid at 30°C. After digestion, suspended solids were removed by centrifugation at 4,000 r.p.m. for 15 minutes. Whole crystal digest supernatants were concentrated by precipitation with 50% ammonium sulphate. The precipitate was re-dissolved in M/100 phosphate buffer pH 8. Digests of solutions of crystals in carbonate/bicarbonate buffer pH 9.5 were collected in M/100 phosphate buffer pH 8 by buffer exchange

on a column of Sephadex G-10 equilibrated in M/100 phosphate buffer pH 8.

Crystal solution digest fractions were concentrated by mixing with dry 'Sephadex' G-10 or G-25.

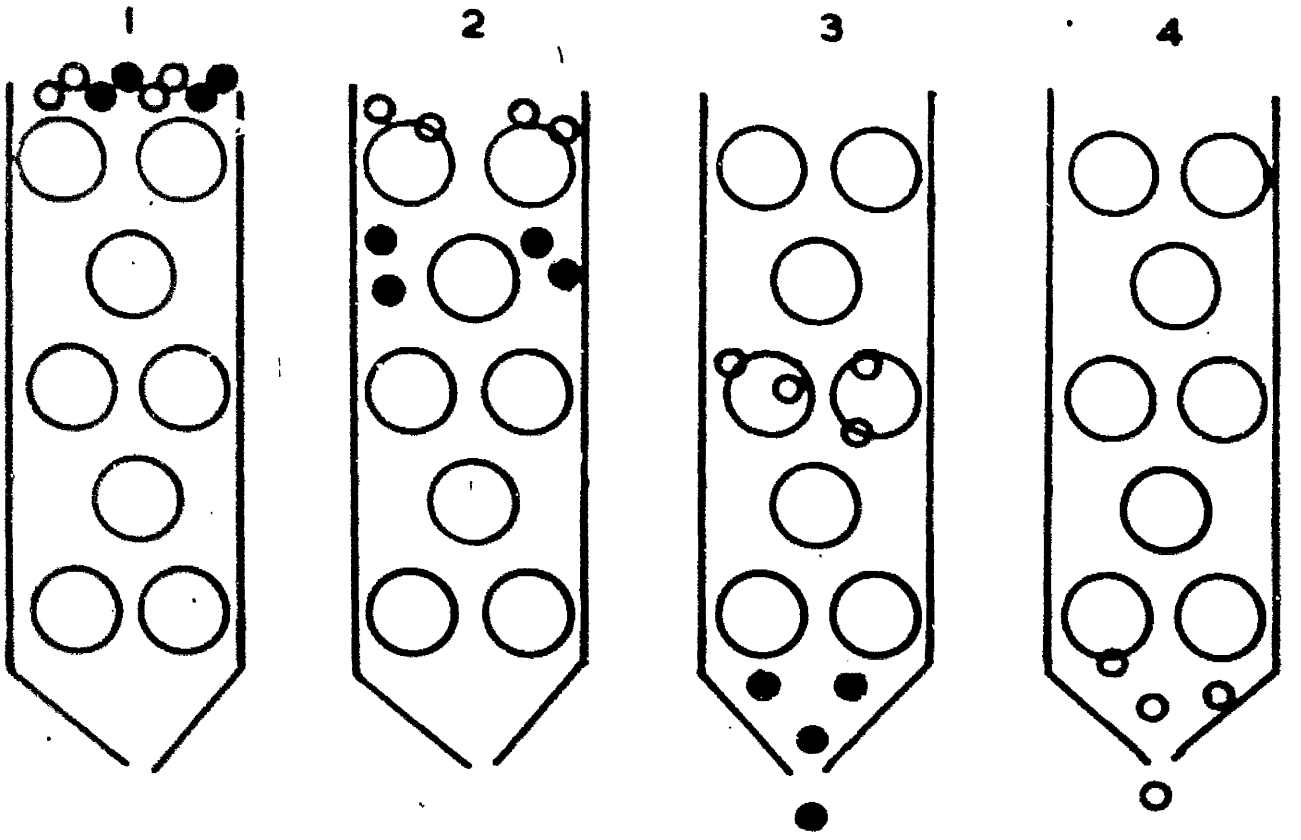
The fraction of the crystal digest for antigenic analysis was prepared by digesting 5-10 mg. of crystals in 1 ml. 0.2M carbonate/bicarbonate buffer at pH 9.5 with 0.2 ml. Pieris brassicae gut juice at 30°C for three hours, followed by removal of undigested crystals by centrifugation at 3,000 r.p.m. for 15 minutes. A secondary digestion was carried out by re-incubation of the supernatant at 30°C for a further hour. The digest antigens were concentrated by precipitation at 50% ammonium sulphate followed by re-dissolving the precipitate in M/100 phosphate buffer at pH 8 at a concentration of 5-10 mg. protein/ml. The significance of the secondary digestion will be explained later.

9. Column Chromatography

(a) Gel filtration with 'Sephadex' - Theory and applications

It is possible to separate mixtures of differently-sized molecules by gel filtration. This is illustrated diagrammatically in fig. 1. Each grade of 'Sephadex' has a particular pore size and Pharmacia (1963) have provided estimations of molecular weight of solutes according to their exclusion from various grades of 'Sephadex'. Also, high molecular weight solutes can be concentrated by mixing with

FIGURE 1 GEL FILTRATION WITH SEPHADEX



SEPHADEX BEADS

● HIGH MOLECULAR WEIGHT MATERIAL – EXCLUDED

○ LOW MOLECULAR WEIGHT MATERIAL – INCLUDED

1. MIXTURE APPLIED TO COLUMN
2. LOW MOLECULAR WEIGHT MATERIAL IS RETARDED
3. HIGH MOLECULAR WEIGHT MATERIAL IS COLLECTED
4. LOW MOLECULAR WEIGHT MATERIAL IS COLLECTED

dry 'Sephadex'. The 'Sephadex' swells by taking up the low molecular weight solutes, e.g. water or buffer. The molecules greater in size than the pores of the gel are not absorbed and so are not concentrated. The 'Sephadex' can then be removed by centrifugation or filtration.

(b) Fractionation of digests

'Quickfit' column CR32/20 was mounted in a 'Beaumaris' fraction collector (Rosemary Lane, Beaumaris, Anglesey, N. Wales) with siphon attachment for collecting volumes of 2-3 ml. 0.5-3 ml. volumes of digests were fractionated on 'Sephadex' grades G-10, G-25, G-50 (fine), G-100 and G-200 equilibrated in M/100 phosphate buffer pH 8.

On collection, fractions intended for toxicity studies were kept at 3°C to prevent loss of toxicity until elution from the column was complete.

Protein content of fractions was estimated from a standard curve of the optical densities of dilutions of a known weight of crystal protein read at 280mμ in the Unicam SP 500 spectrophotometer (Unicam Instruments Ltd., Cambridge). The table over gives the readings of optical densities of dilutions of a known weight of crystal protein. The results are expressed graphically in fig. 2 and this graph was used for estimating protein concentration of material of known optical density.

FIGURE 2 CALIBRATION CURVE OF CRYSTAL PROTEIN AT 280 Mμ

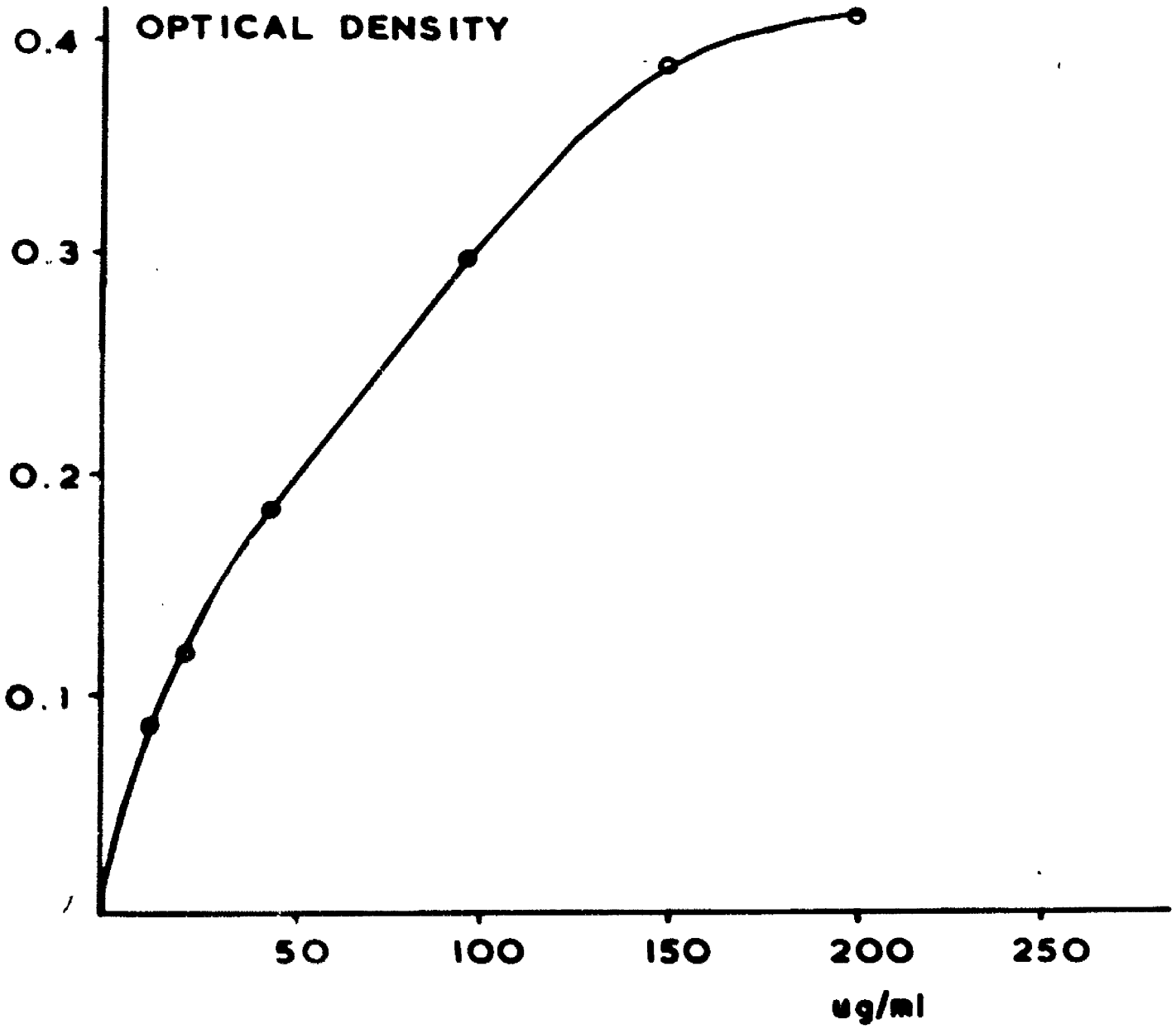


Table 9

Calibration of spectrophotometer at 280 mμ - optical density of crystal protein solutions

CONCENTRATION OF CRYSTAL PROTEIN (μg/ml.)	OPTICAL DENSITY
10	0.09
25	0.125
50	0.18
100	0.3
150	0.39
200	0.41

10. Antigenic Analysis of Crystal Solutions and Crystal Digests

(a) Double diffusion in agar

Agar gels consisted of 1% (w/v) 'Ionagar' No.2 (Oxoid Division of Oxo Ltd., London, S.E.1.) + 0.25% (w/v) sodium chloride + 1/10,000 thiomersalate. Glass Petri dishes (10 cm. in diameter) were coated with 0.25% (w/v) 'Ionagar' No.2 and allowed to dry. 20 ml. of gel was used to pour each plate. Gel punches were used to cut circular wells in the agar. The diameters of the wells and their distances apart varied according to the potencies of the antisera which were used undiluted. Antigens were used at a concentration of 5-10 mg. protein/ml. When antigens and antisera had been placed in the wells, the plates were incubated at 37°C in plastic air-tight boxes containing moistened cotton wool to prevent drying of the gel. Gels

were examined at intervals of several hours to follow the development of precipitin lines. The incubation time was rarely extended beyond twenty-four hours. The gels were removed from the Petri plates and washed in several changes of slightly alkaline (pH 7.4) saline solution to remove antigen and serum which had not been fixed by specific reaction. The washed gels were stained by immersion in a solution of naphthalene black in 5% acetic acid until the stain had diffused throughout the whole gel which required from 30 minutes to 1 hour. After staining, the gels were de-colourised (24-72 h.) in 5% acetic acid to reveal the blue-black stained precipitin lines. A permanent record of the immunodiffusion reaction was made by completely drying the gel in an incubator at 37°C on a lantern slide glass cover previously coated with a dried thin layer of Ionagar solution to prevent shrinkage and cracking of the gel. Photographic records were made by placing the stained slide in the negative carrier of a photographic enlarger and enlarged projection prints made.

(b) Immunoelectrophoresis in agar and polyacrylamide:

Agar gels consisted of 1% (w/v) 'Ionagar' No.2 + 'Oxoid' (Oxoid Division of Oxo Ltd., London, S.E.1.) barbitone-acetate buffer at a concentration of 8.26 g./l. + 1/10,000 thiomersalate. Glass lantern slide cover glasses, 8 cm. square, were coated on one side with 0.25% (w/v)

Ionagar No.2 and allowed to dry. The slides were poured with 12-15 ml. of molten electrophoresis agar and allowed to set in a horizontal position. Antigen wells and antiserum trenches were cut with a 'Buchler' gel cutter (Buchler Instruments, Fort Lee, N.J., U.S.A.). Antigen preparations to be electrophoresed were placed in the wells. A drop of 1% bromothymol blue was placed in a separate well as an indicator of the progress of the electrophoresis. The tank buffer consisted of 18.5 g. of boric acid + 2 g. of sodium hydroxide + 1l. distilled water. Contact between tank buffer and electrophoresis agar was made with lint wicks and electrophoresis was carried out at a potential drop of 10 volts/cm. The rate of migration was estimated from the position of the dye spot and electrophoresis was terminated when the dye spot was about 1 cm. from the wick contact. The agar in the cut antiserum trenches was removed and the trenches were filled with antiserum. The gels were then incubated at 37°C in an air-tight box to prevent drying of the gels. Gels were washed and stained when the precipitin lines had developed to the desired extent.

Electrophoresis of antigen preparations in polyacrylamide gels was carried out in perspex trays 0.7 cm. deep and 12.5 cm. broad by 14 cm. long after the method described by Baillie - (1964). A close-fitting lid carried 8 slot formers which were also of perspex 1 mm. thick and 1 cm. long. The gel was

prepared by catalytic polymerisation of the monomer 'Cyanogum' 41 (B.D.H.) in 'Tris'-citrate buffer or barbitone-acetate buffer (Oxoid). The 'Tris'-citrate buffer was prepared by dissolving 4.59 g. 'Tris' and 0.53 g. citric acid in 1 litre of distilled water. 1.5 ml. of 10% aqueous solution of ammonium persulphate and 1.5 ml. of 10% aqueous solution of dimethylaminoethylcyanide as catalysts were added to 150 ml. of 7% 'Cyanogum' and the mixture was evacuated thoroughly to remove dissolved air. It was then poured into the perspex tray and the lid was lowered gently into position, care being taken to avoid formation of air bubbles. Polymerisation took place in the absence of oxygen and was complete after one hour at room temperature. The gel was then ready for use. The slots which formed were filled with the antigen preparations to be electrophoresed and soft white paraffin (paraffinum molle album, B.D.H.) was melted and poured onto the sample slots to prevent cross-leakage between the slots. 'Melinex' 'O' film, gauge 50 (polyester film, I.C.I.) was used to cover the gel during electrophoresis. Contact between gel and tank buffers was made with absorbent lint wicks and the gel was run with a potential drop of 10 volts/cm. A brown line artefact developed at the interface of the two buffers and migrated towards the anode. The electrophoresis was terminated when the brown line had travelled 10 cm. from the row of sample

slots. The gel was cut into three horizontal slices by a fine suture wire held taut between the fingers. The thickness of the slices was kept constant by means of perspex guide strips. The centre slice was placed on a sheet of perspex for immunodiffusion. Cuts were made in the polyacrylamide parallel to and slightly overlapping the electrophoresed preparation. This section between the slots was discarded and replaced with 1% Ionagar No.2 as used for immunodiffusion. After solidification, an antiserum trench was cut in the centre of the agar block and filled with antiserum. The gel was then incubated on the perspex sheet in an air-tight box at 37°C to allow the development of precipitin bands.

The top or bottom slice of the same polyacrylamide gel was stained for protein by naphthalene black in 5% acetic acid overnight and de-colourised by several washes in 5% acetic acid. The protein bands which were then evident in the gel electrophoretic runs were compared with the precipitin bands. It was thus possible to correlate protein bands and precipitin arcs.

11. Column Chromatography of Berliner Crystal Digest Fraction C2 + Specific Antisera

Antisera A and I and normal rabbit serum were precipitated at 50% ammonium sulphate concentration and the precipitate containing the immuneglobulins was re-dissolved

in a minimum of M/100 phosphate buffer at pH 8. The solutions of partially-purified globulins were fractionated on a column of 'Sephadex' G-50 and the excluded material was retained and re-concentrated by precipitation at 50% ammonium sulphate concentration. Aliquots of about 500 µg. of fraction C2 of the crystal digest of berliner crystals were mixed with each purified antiserum and normal rabbit serum so that the antigen a was no longer detectable in gel diffusion in the mixture of fraction C2 + antibody A and likewise for antigen i with antiserum I.

The mixtures (1) antibody A + fraction C2, (2) antibody I + fraction C2 and (3) normal rabbit antibody + fraction C2 were passed through a column of 'Sephadex' G-50 and the pooled, included fractions were retained for estimation at 280 mµ for protein content and for antigenic analysis by gel diffusion. Fractions were re-concentrated by precipitation with 50% ammonium sulphate.

12. Electrophoretic Esterase Analysis

Esterase analysis involved electrophoresis in starch gels after the technique described by Norris - (1964) and Norris & Burges - (1963) of vegetative cell disintegrates which were prepared as described in section 4. Gel moulds were glass plates to which were attached glass strips to form a rectangle 10 cm. x 15 cm. interior measurements. Joints were sealed with silicone stopcock grease (Edwards

High Vacuum Co., Ltd.) and the inside of the mould was smeared with a thin film of silicone grease to allow the starch gel to be removed without adhesion to the glass.

15 g. of starch (Hydrolysed Starch - Connaught Medical Research Laboratories) was mixed with 120 ml. 'Tris'-citrate buffer in a 500 ml. conical flask and heated gently over a bunsen flame with continuous swirling. The solution first thickened, then liquified. When boiling began, heat was discontinued and bubbles were removed by partially evacuating the flask and the vacuum was suddenly released so that the bubbles collapsed. The molten gel was poured into the tray and immediately covered with a sheet of 'Melinex' film. After two hours at room temperature, the gel had solidified and cooled sufficiently for use. The gel was cut across approximately 3 cm. from one end and the short end section was pulled back gently to expose the cut surface. Cell extracts for analysis were soaked on to 1 cm. lengths of Whatman's No.3MM fiter paper strip (W. & R. Balston Ltd.) as used for paper chromatography. The strips were applied to the cut surface of the gel and the short section of the gel was replaced to close the cut completely. The 'Melinex' cover was replaced and, for electrophoresis, contact was made between the tank buffer and the gel with absorbent lint wicks. The tank buffer was borate buffer as used in section 10. A

potential drop of 10 volts/cm. was maintained along the gel during electrophoresis. As in polyacrylamide, a brown line artefact developed which was allowed to travel 10 cm. from sample insert, then electrophoresis was discontinued. The starch gel was then cut into three horizontal slices by the same method described for polyacrylamide gels. The middle slice was retained for esterase staining. This slice was immersed in 60 ml. 0.1M 'Tris' + 40 ml. 0.1M maleic acid + 2 ml. 1% α-naphthyl acetate in 50% acetone + 50 mg. fast blue BB salt. Esterase bands developed as red areas in the gel and reached maximum intensity in about two hours at room temperature.

13. Insect Rearing Conditions

A continuous supply of insect larvae was required for toxicity tests of materials derived from the toxic crystals and for the extraction of gut juice to study digestion of the crystals in vitro.

(a) Pieris brassicae

David & Gardiner - (1952) have shown that this insect could be reared continuously throughout the year in the laboratory without hibernation taking place if the larvae were illuminated for at least sixteen hours out of twenty-four. They also showed that successful matings could be obtained with a combination of artificial light and daylight or either

alone.

A supply of ova was obtained from Dr. David, A.R.C. Unit of Insect Physiology, Cambridge. Young larvae, up to the commencement of the third instar (growth stage), were reared on fresh cabbage in plastic sandwich-boxes with the lids partially open to prevent condensation and growth of moulds. When the larvae were large enough to handle individually with forceps, they were transferred to cabbage into shallow 12" x 24" plastic gravel trays covered with muslin to allow air to circulate freely. The death rate was higher when deeper boxes were used.

Larvae pupated on the sides of the trays and sometimes on the muslin lid. Adults emerged in the trays and were transferred to muslin-covered cages which were placed in an east-facing window. Daylight alone was not sufficient under these conditions to allow mating of the butterflies and the natural illumination was supplemented by 1-3 150 watt lamps. This was sufficient to maintain the culture although there were fewer matings than had been obtained previously with the cage placed in a greenhouse in summer.

The butterflies were fed a 25% solution of sucrose or honey on a yellow or purple cotton-wool pad. Cabbage leaves were placed on the bottom of the cage for egg-laying.

All stages of the insect were reared in the laboratory at room temperature (22-27^{o}C).

(b) Philosamia cynthia

A supply of ova of this silk-moth was obtained from Worldwide Butterflies Ltd., Charmouth, Bridport, Dorset. Young larvae were fed privet (Ligustrum vulgare) in air-tight sandwich boxes and were sprayed with water daily to maintain a humid atmosphere which is beneficial to this insect. Older larvae were transferred to gravel trays which had glass lids to prevent drying of the foodplant. Pupae were formed in cocoons on the bottom of the trays and were removed after three days and placed in a muslin-covered cage. Adults emerged after about one month and eggs were deposited on the sides of the cage in loose clumps. The eggs were collected and kept in air-tight boxes.

Adults of this moth did not require any lighting for mating which took place at night. Also the adults were unable to feed and so no sugar was supplied. When privet was supplied, the moths did not lay their eggs on it in preference to the muslin.

14. Toxicity Tests on Insects

Larvae of Pieris brassicae were used for determining toxicity of various fractions of digests of crystals and of soluble crystal extracts. Samples were administered by ingestion and injection at a concentration and dosage sufficient to have given 100% mortality when whole crystals

or unfractionated digest was administered in control experiments. All samples were in solution in M/100 phosphate buffer pH 8 and were sterilised by filtration through a glass filter grade 5/3.

(a) Ingestion

Discs were cut out of young cabbage leaves with a cork-borer giving a disc of diameter about 1 cm. One of the surfaces of the disc was shaved off with a sterile scalpel and the sample was pipetted on to the exposed surface to cover it evenly. If the surface was not removed it was difficult to distribute the sample evenly over the surface due to its water-repellant nature. When the sample had dried, 8-10 first or second instar larvae were introduced on to the surface of the cabbage disc which was kept in an air-tight box 1" x 1" x 2" to prevent further drying. The number of dead or dying larvae were counted after 24 hours and again after 48 hours.

(b) Injection

Final instar larvae (5th) were injected into the haemocoel (body cavity) at a point approximately mid-way along the length of the larva. Approximately 0.02 ml. of the sample at a concentration of 150 µg./ml. was taken up in a Pasteur pipette which had been drawn out to a very fine short capillary. The sample was expelled by pressure on a rubber bulb attached to the upper end of the pipette. Control

experiments showed that not more than 10% of larvae thus injected with M/100 phosphate buffer pH 8 were killed. After injection, the larvae were placed on fresh cabbage which was replaced regularly and the dead larvae were counted after three and seven days.

Preparations intended for toxicity tests were stored at -20°C.

RESULTS

TABLE OF CONTENTS

1. Preparation Methods for Crystals of B. thuringiensis Isolates

Crystals were required for (1) preparation of antisera to spore-free crystals so that the antisera would contain no antibodies to spore antigens and (2) crystal digestion experiments where the presence of spores might interfere with or complicate the digestion of the crystals.

The method of Bateson - (1965) for isolating crystals was early considered to be time-consuming and to give very small yields of crystals. Attempts were made at various times throughout the work of this thesis to shorten the method of Bateson - (1965) and to increase crystal yield while maintaining the crystal purity at 98-99%. The methods described on p.36 represent a step-wise improvement in the design of the phase separation technique. The latest method (d) was the most satisfactory. Method (c) was applied to one strain, dendrolimus, where (a) and (b) were unsatisfactory. The results which follow demonstrate how these methods developed and the factors which influenced their selection as suitable methods for preparing crystals.

(a) Effect of proportion of aqueous phase to organic phase in phase separation

Preliminary experiments showed that the ratio of organic phase to aqueous phase in the phase separation process, as described on p.38 , affected the yield or

recovery of crystals although it did not affect the purity of the crystals. Fig. 3 shows the yields of crystals obtained from a suspension of berliner spores and crystals after flotation as described on p. 38 (spore:crystal ratio 20:80). The volumes were as follows:-

(1) 10 ml. spore/crystal suspension + 10 ml. carbon tetrachloride + 80 ml. 1% Na_2SO_4.

(2) 20 ml. spore/crystal suspension + 20 ml. carbon tetrachloride + 60 ml. 1% Na_2SO_4.

(3) 25 ml. spore/crystal suspension + 25 ml. carbon tetrachloride + 50 ml. 1% Na_2SO_4.

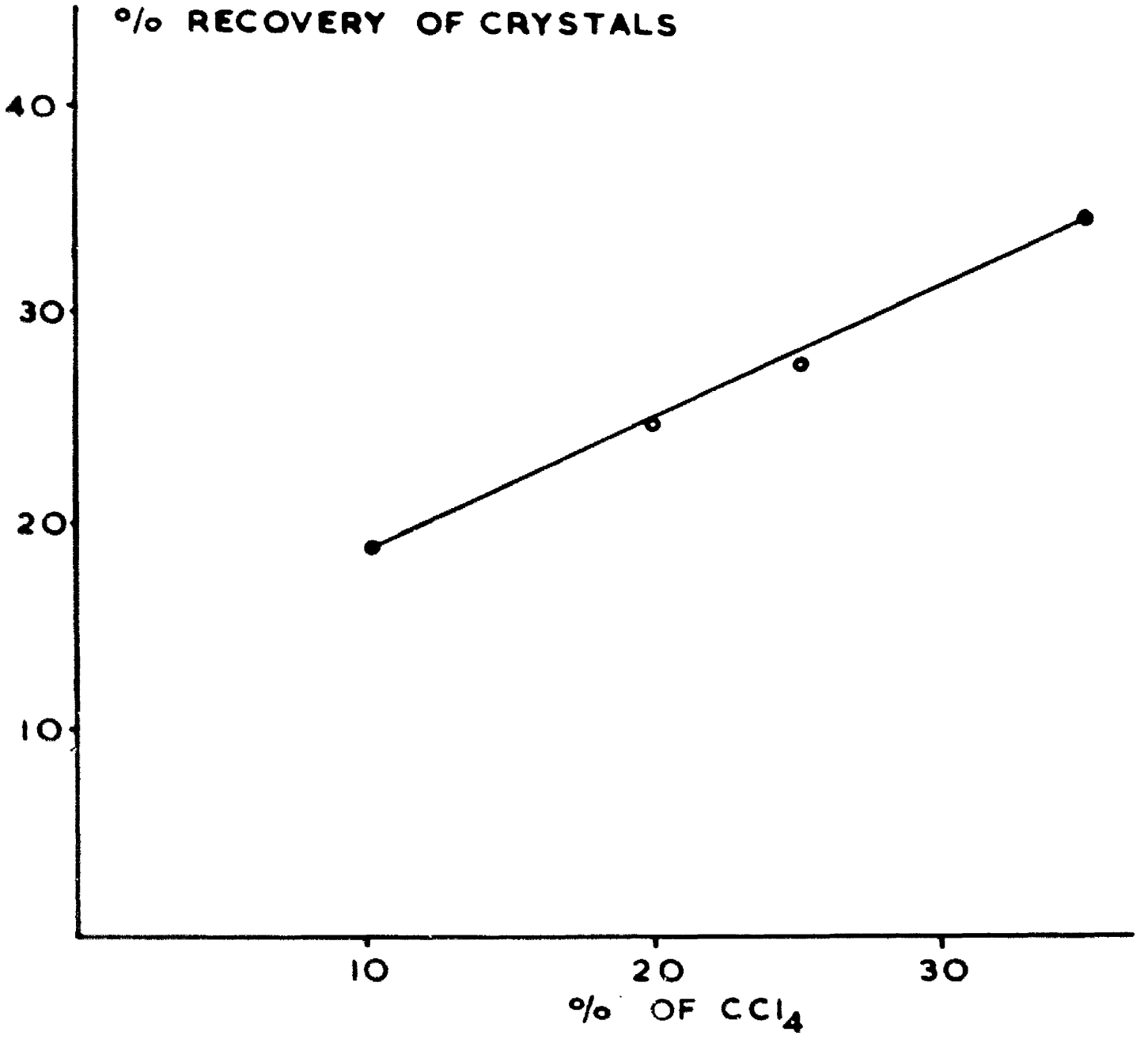
(4) 35 ml. spore/crystal suspension + 35 ml. carbon tetrachloride + 30 ml. 1% Na_2SO_4.

From the graph (fig.3) the yield of crystals per unit volume of the original suspension increases in linear proportion to the volume of the organic solvent. The yield of crystals was not determined beyond the ratio 35:35:30 since the emulsion which contained more than 35 ml. of organic solvent was more difficult to separate to obtain crystals from the aqueous phase. The ratio 35:35:30 was thus selected for giving optimum yields of crystals (35% recovery).

(b) Effect on crystal yields and purities of spore flotation

A further variable which affected both yield and purity of crystals by phase separation was the ratio of spores to crystals in the suspension. As described on p.38, the spores could be selectively removed from the original 50:50 spore/crystal mixture so that the percentage of spores in the suspension could be reduced to about 20%. When a

FIGURE 3 RECOVERY OF CRYSTALS FROM ORIGINAL SUSPENSION OF 80% CRYSTALS AND 20% SPORES BY PHASE SEPARATION WITH VARIOUS % OF CCl_4



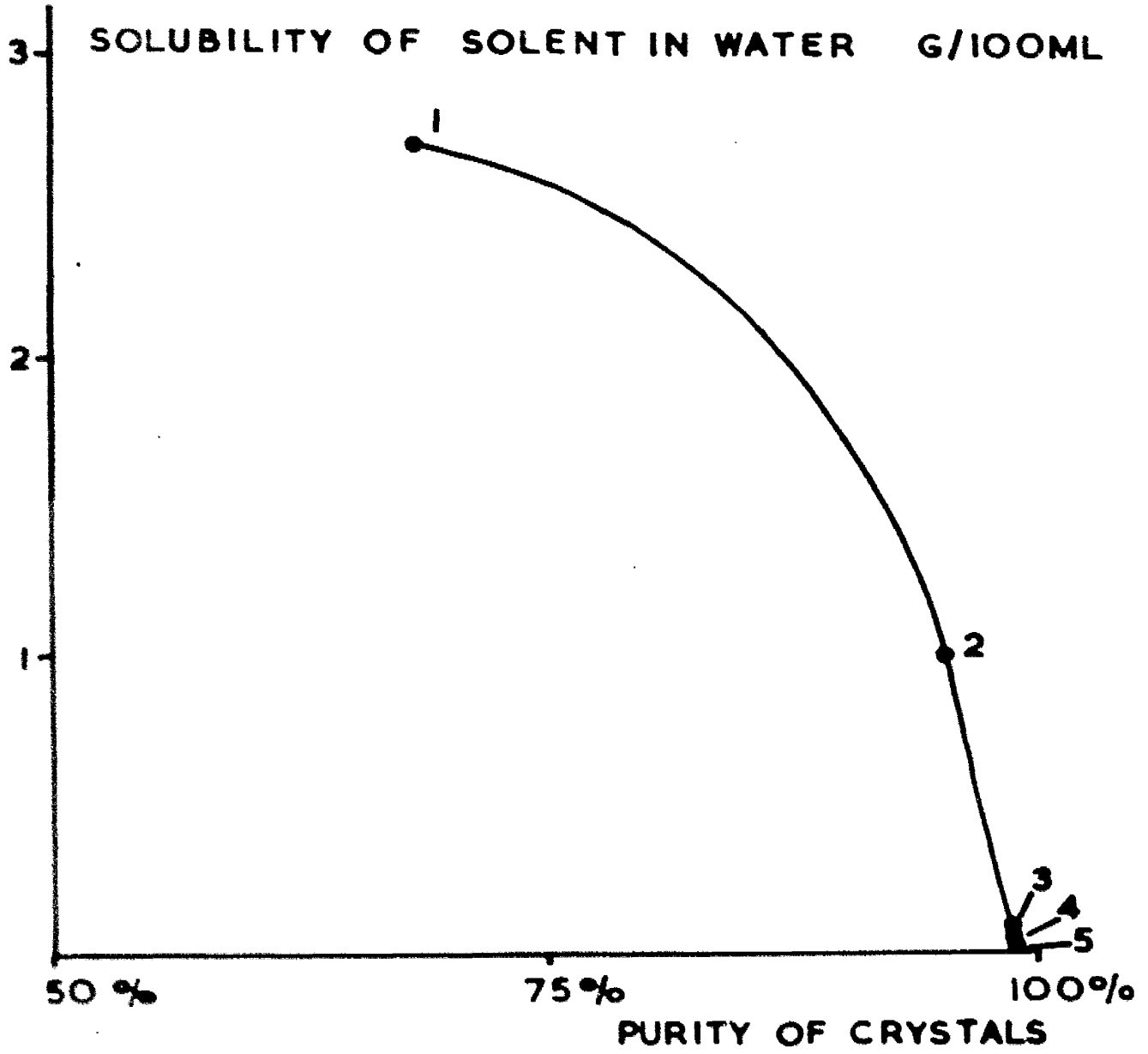
20:80 spore/crystal suspension of the berliner strain was phase separated by method (b), the yield of crystals was 35% at a purity of 98-99%. By the same method but without the preliminary flotation, i.e. with a suspension containing 50:50 spores/crystals, the yield of crystals was only 12-15% at a purity of 96%.

In most strains repeated spore flotation was only able to lower the spore percentage to about 20%. However, in some strains, notably those of the kenya group, (defined on p.31) the flotation process readily reduced the spore count so that the resulting suspensions contained about 99% crystals after repeated frothing. These suspensions could not be purified further by phase separation since homogenisation of these suspensions with xylene resulted in the crystals and the small number of spores being taken up together by the xylene.

(c) Effect on crystal purity of solubility of the organic solvent in water

Examination of a range of solvents of low solubilities in water showed that there was a relationship between the solubility of the solvent in water and the purity of the crystals obtained by phase separation with the solvent. The highest crystal purities were obtained with solvents of lowest water solubility. These results are shown in fig.4. From this graph, it was decided that xylene gave a

FIGURE 4 EFFECT ON CRYSTAL PURITY OF SOLVENTS OF DIFFERENT WATER-SOLUBILITIES USED IN PHASE SEPARATION



1 - AMYL ALCOHOL

2 - CHLOROFORM

3 - CARBON TETRACHLORIDE

4 - XYLENE

5 - PARAFFIN

satisfactorily high crystal purity. Paraffin oil gave equally good results but was too viscous to allow good separation of the emulsion.

(d) Comparison of methods used for preparing crystals

The methods (a), (b), (c) and (d) for the preparation of crystals were described on p.36 and the purities and yields of crystals are tabulated below

Table 10

Crystal purities and yields obtained by methods (a), (b), (c) and (d)

METHOD	SOLVENT	CRYSTAL PURITY	CRYSTAL YIELD
(a) 2 successive phase separations modified (Bateson - 1965)	CCl_2F-$CClF_2$	96% after 1st treatment 98% after 2nd treatment	12-15% approx.2-3%
(b) flotation then phase separation	CCl_4	80% after flotation 98-99% after phase sepn.	 25-45%
(c) 1 phase separation (dendrolimus)	CCl_4	96%	not known
(d) flotation then phase separation	xylene	80% after flotation +99% after phase sepn.	 50-65%

2. Antigenic Analysis of Alkali Extracts of Crystals

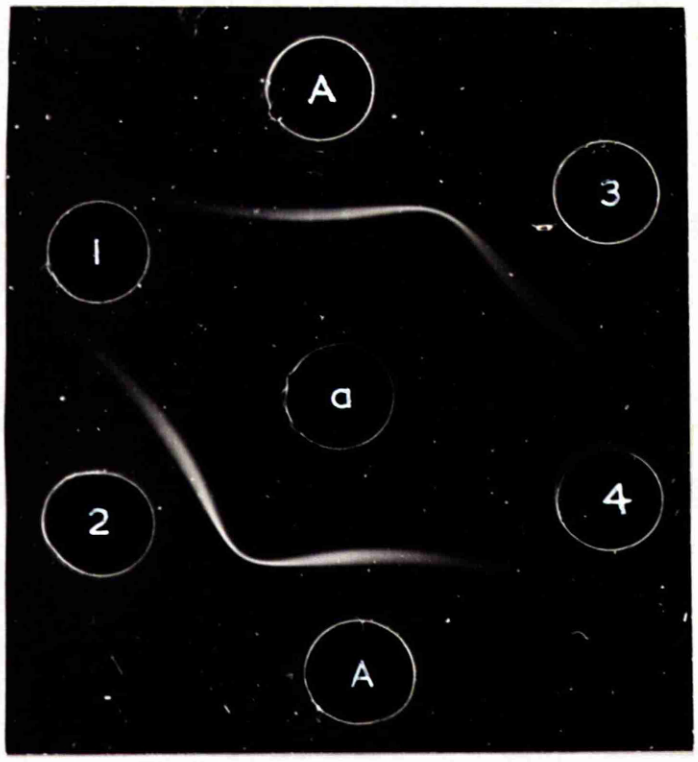
(a) Selection of antisera for use in antigenic analysis

Antisera were prepared to suspensions of 98-99% pure crystals prepared from 23 strains of crystal-formers as described on p.43. When each of these antisera were diffused in agar gel against an alkali extract of the immunising crystals, i.e. the homologous system, one strong precipitin line and in most cases one or more fainter, more diffuse lines were formed. Attempts were made to include consideration of these diffuse lines in the antigenic analysis but were not successful for two reasons; firstly, the diffuse lines were not always detectable and varied from one gel to another, and, secondly, they were so diffuse and weak that the normal criteria of identity and non-identity could not be applied to them. For these reasons only the main line was considered in antigenic analysis.

The twenty-three antisera were compared with each other by diffusing antisera X and Y against antigen x and noting whether the main precipitin line between X and x was continuous or not with the reaction, if any, between antiserum Y and antigen x. This principle is shown in fig.5 and fig.6 and Plates 1 and 2. Out of the twenty-three antisera prepared it was possible to distinguish eleven different types on the basis of continuity or discontinuity of their main precipitin line. The eleven

PLATE I

CRITERIA OF SPECIFICITY OF ANTISERA

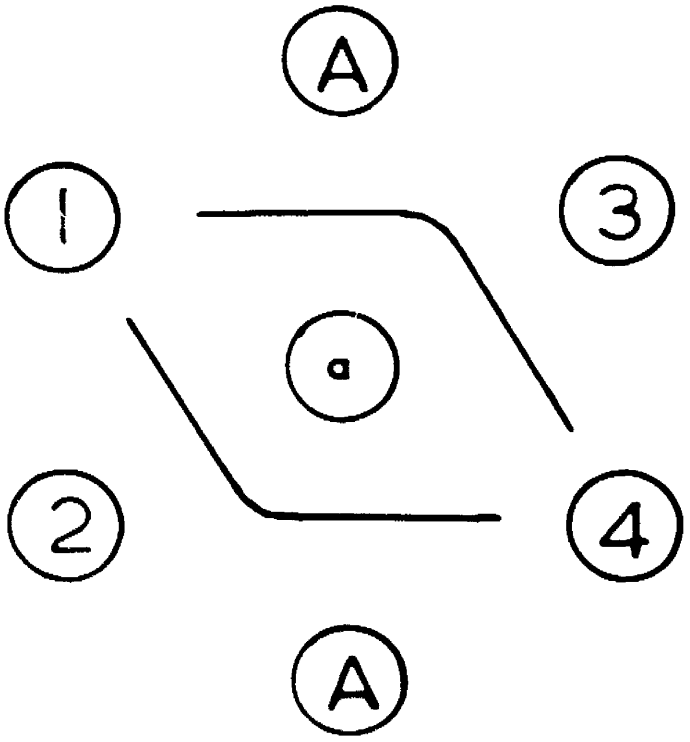


A - antiserum to <u>berliner</u> crystals
a - solution of <u>berliner</u> crystals
1 - antiserum to T 84-A crystals
2 - antiserum to <u>subtoxicus</u> crystals
3 - antiserum to Limassol crystals
4 - antiserum to Tolworth crystals

Antisera 2 and 3 give reactions to antigen <u>a</u> identical to that of antiserum A to antigen <u>a</u>.

Antisera 1 and 4 differ from antiserum A.

FIGURE 5 CRITERIA OF SPECIFICITY OF ANTISERA



A - ANTISERUM TO BERLINER CRYSTALS

a - SOLUTION OF BERLINER CRYSTALS

1 - ANTISERUM TO T84-A CRYSTALS

2 - ANTISERUM TO SUBTOXICUS CRYSTALS

3 - ANTISERUM TO LIMASSOL CRYSTALS

4 - ANTISERUM TO TOLWORTH CRYSTALS

ANTISERA 2 & 3 GIVE REACTIONS TO ANTIGEN IDENTICAL TO THAT OF ANTISERUM A TO ANTIGEN

ANTISERA 1 & 4 DIFFER FROM ANTISERUM A

PLATE 2 CRITERIA OF IDENTITY OF ANTISERA

1 - subtoxicus crystal solution

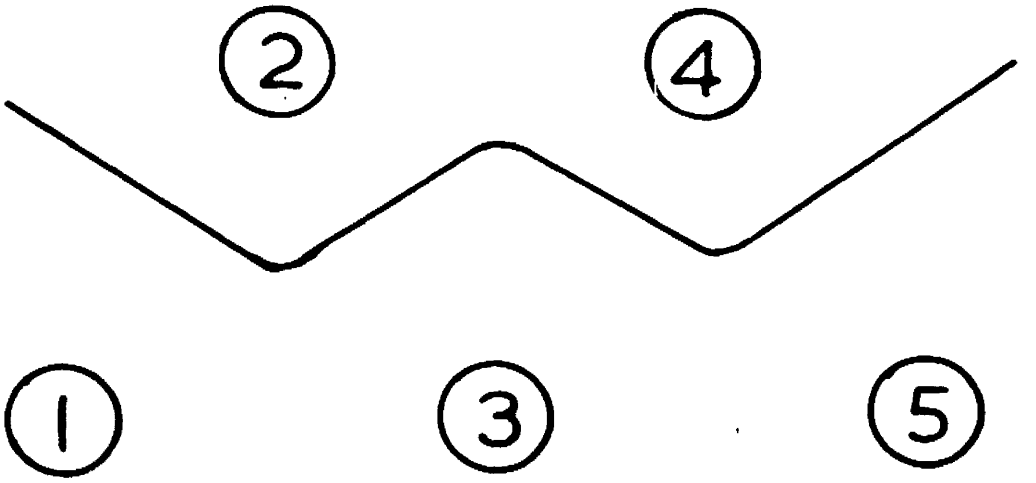
2 - antiserum to subtoxicus crystals

3 - berliner crystal solution

4 - antiserum to berliner crystals

5 - subtoxicus crystal solution

FIGURE 6 CRITERIA OF SPECIFICITY OF ANTISERA



1 – SUBTOXICUS CRYSTAL SOLUTION

2 – ANTISERUM TO SUBTOXICUS CRYSTALS

3 – BERLINER CRYSTAL SOLUTION

4 – ANTISERUM TO BERLINER CRYSTALS

5 – SUBTOXICUS CRYSTAL SOLUTION

different main precipitin lines thus represented eleven different antigen/antibody systems since each antiserum, when diffused against a solution of its immunising crystals, behaved as a monospecific antiserum when the faint, diffuse lines were not under consideration. The eleven antisera types were labelled from A to K and their corresponding antigens from a to k. The table below describes the original twenty-three homologous reactions.

Table 11

Antisera types prepared by immunisation with whole crystals from 23 isolates of crystal-formers

ISOLATE STRAINS	ANTIGEN	ANTISERUM	PRECIPITIN LINES IN GEL
*berliner	a	A	one line
anduze	a	A	one line,weak line nr.antigen well
Limassol	a	A	one line
subtoxicus	a	A	one line
3625	a	A	one line,weak line nr.antigen well
3626	a	A	one line,weak line nr.antigen well
*finitimus	b	B	one line,weak line nr.antigen well
*sotto (T84-A)	c	C	one line
1 H-A	c	C	one line
*dendrolimus	d	D	one line,weak line nr.antiserum well
*Kenya	e	E	one line,weak line nr.antiserum well
Tolworth	e	E	one line
E1	e	E	one line
*G.1	f	F	one line,weak line nr.antiserum well
PIL 106	f	F	one line,weak line nr.antigen well
*Morrison	g	G	one line,weak line nr.antiserum well
*77MD0559 (alesti)	h	H	one line,weak line nr.antiserum well
T63L4	h	H	one line,weak line nr.antigen well
*galleria allemand	i	I	one line,2 weak lines nr.antigen well
*PIL 105	j	J	one line
plodia interpunctella	j	J	one line
*P1	k	K	one line
Kilmarnock	k	K	one line

* Indicates 11 different reactions used to type 94 isolates.

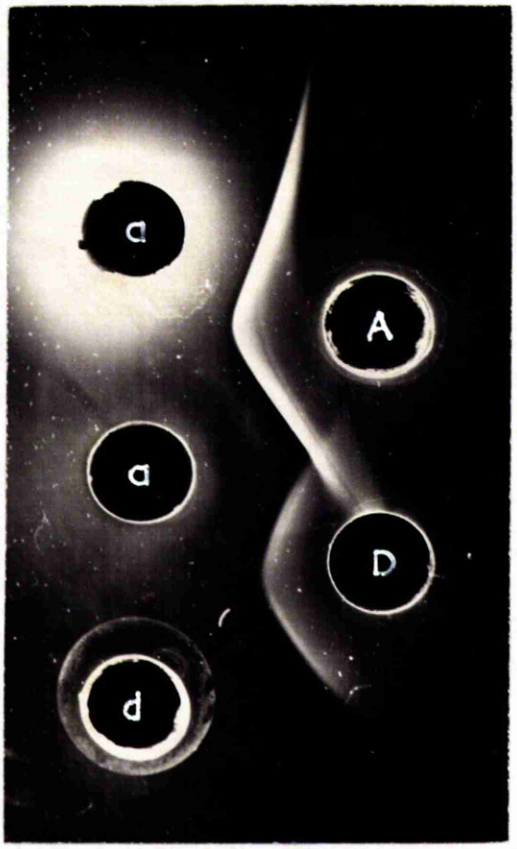
It should be explained that, originally, strains representative of the twelve sub-groups of Norris - (1964) were selected for the preparation of antisera and some of the antisera were not prepared until a later stage. It was found that several crystal extracts gave no reactions of identity with any of the main lines of homologous reactions of the antisera available at the time. Therefore, antisera were prepared to crystals from these non-reacting strains. Eleven different antisera corresponding to the eleven different antigens or main precipitin lines were finally available and all of the 94 strains gave a reaction of identity with at least one of the eleven different antigens.

(b) Behaviour of antisera diffused against heterologous crystal solutions

Although only a single antibody was produced in quantity when rabbits were immunised with whole crystals, solubilisation of the crystals released other antigens. Thus in Plate 3 and fig.7, antiserum A contains a single antibody reacting with antigen a present in a solution of berliner crystals; it does not contain antibody D. However, a solution of berliner crystals contains antigen d in addition to antigen a and will, therefore, react with a serum containing antibody D.

Soluble alkali extracts were prepared from all the ninety-four isolates of crystal-formers in the collection and

PLATE 3 REACTION OF A CRYSTAL SOLUTION TO TWO DIFFERENT ANTISERA



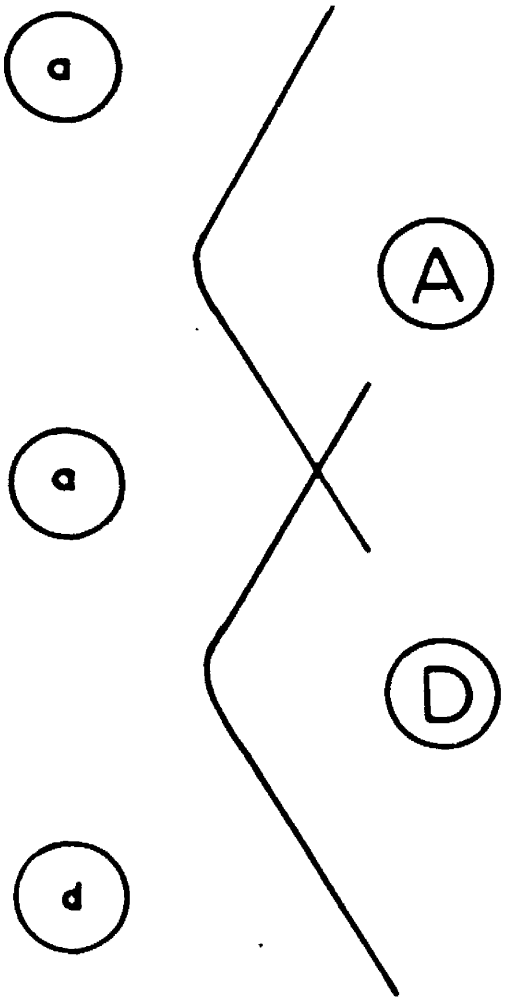
a - solution of berliner crystals

A - antiserum to berliner crystals

d - solution of dendrolimus crystals

D - antiserum to dendrolimus crystals

FIGURE 7 REACTION OF A CRYSTAL SOLUTION TO 2 DIFFERENT ANTISERA



a — SOLUTION OF BERLINER CRYSTALS

A — ANTISERUM TO BERLINER CRYSTALS

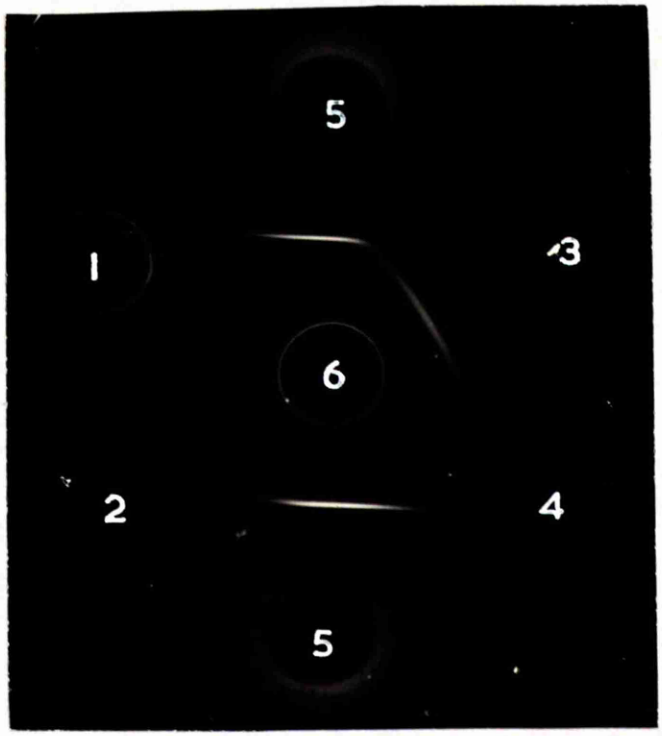
d — SOLUTION OF DENDROLIMUS CRYSTALS

D — ANTISERUM TO DENDROLIMUS CRYSTALS

were diffused against each of the eleven different antisera using homologous antigens as controls for determining reactions of identity and non-identity between test and control antigens. The criteria for determining reactions of identity and non-identity are illustrated in fig.8 and Plate 4. The gel diffusion patterns obtained by diffusing four of the ninety-four isolates against the eleven control reactions are shown in Plate 5 and fig.9.

One further point requires mention. Some of the eleven antisera apparently contained antibody types other than those corresponding to antigens in the solutions of the immunising crystals. For example, antiserum C prepared by immunisation with T 84-A crystals gave only one precipitin line when diffused against a solution of T 84-A crystals and this precipitin line corresponded to the reaction between antibody C and antigen c. When other crystal solutions, those containing the antigen e, were diffused against this antiserum a precipitin line was formed which crossed the precipitin line C/c. This is shown in Plate 6 and fig.10. Here the C/c line has not formed a barrier to the other precipitin line and so the two precipitin lines were due to different antibodies C and E. This interpretation will be discussed later when it will be suggested how antigens other than those present in the immunising crystals could elicit antibody formation. This phenomenon is distinct from the weak

PLATE 4 CRITERIA OF IDENTITY AND NON-IDENTITY OF ANTIGENS



1,2,3,4 - test antigens

5 - control antigen

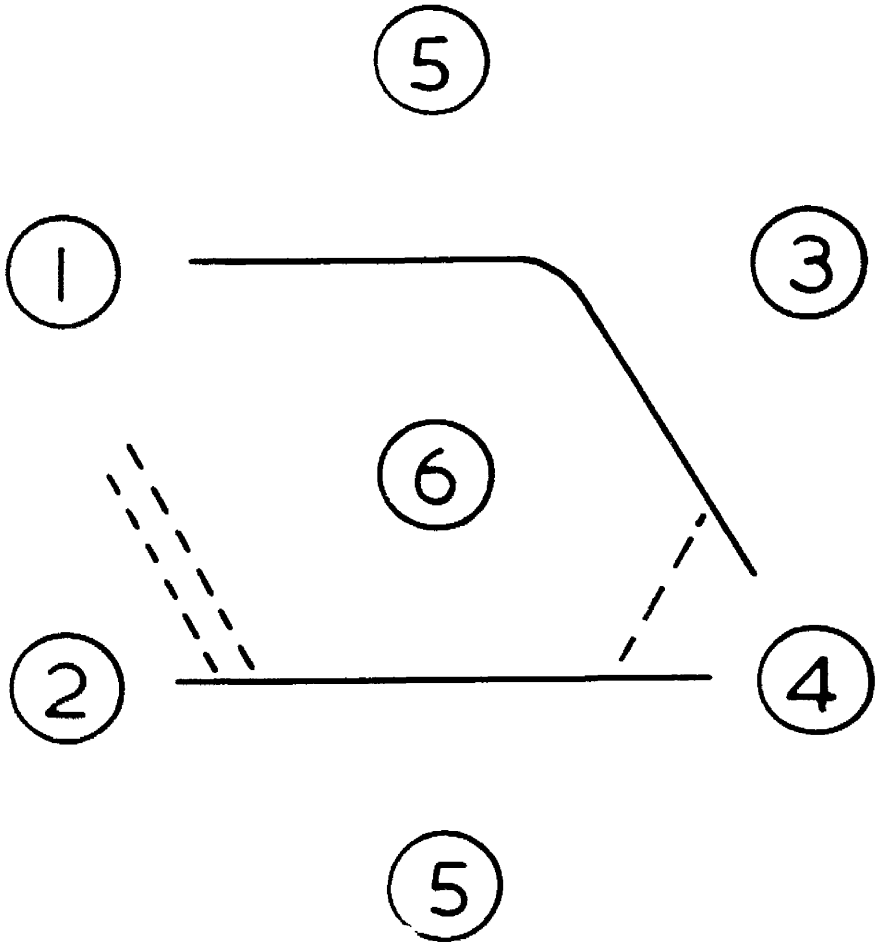
1 - no reaction negative

2,4 - non-identity negative

3 - identity positive

6 - antiserum to antigen 5

FIGURE 8 CRITERIA OF IDENTITY AND NON-IDENTITY OF ANTIGENS



1, 2, 3, 4 — TEST ANTIGENS

5 — CONTROL ANTIGEN

1 — NO REACTION NEGATIVE

2, 4 — NON-IDENTITY NEGATIVE

3 — IDENTITY POSITIVE

6 — ANTISERUM TO ANTIGEN 5

PLATE 5

DIAGRAMMATIC ILLUSTRATION OF REACTIONS OF CRYSTAL SOLUTIONS OF FOUR ISOLATES WITH THE ELEVEN DIFFERENT ANTISERA

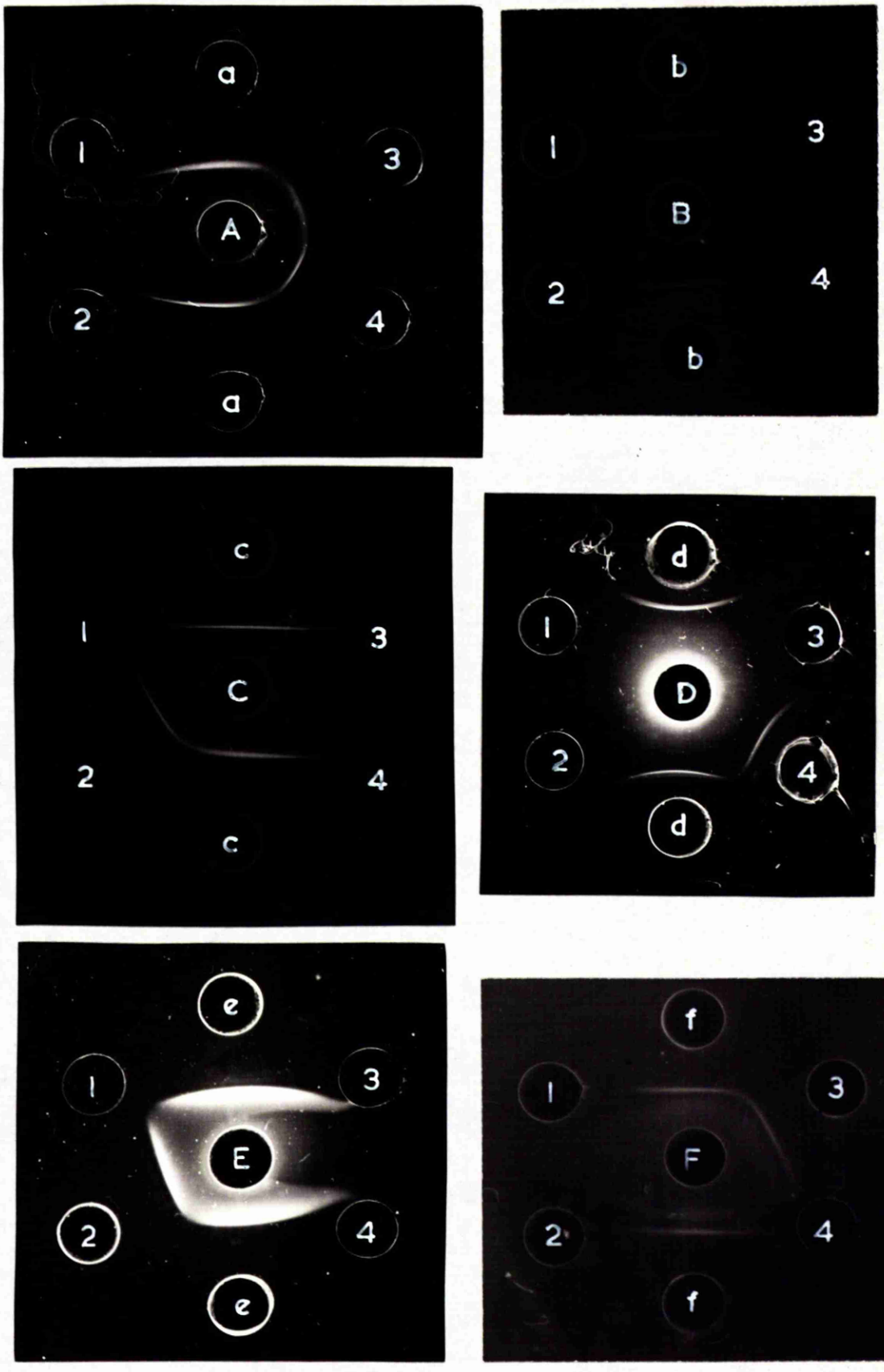
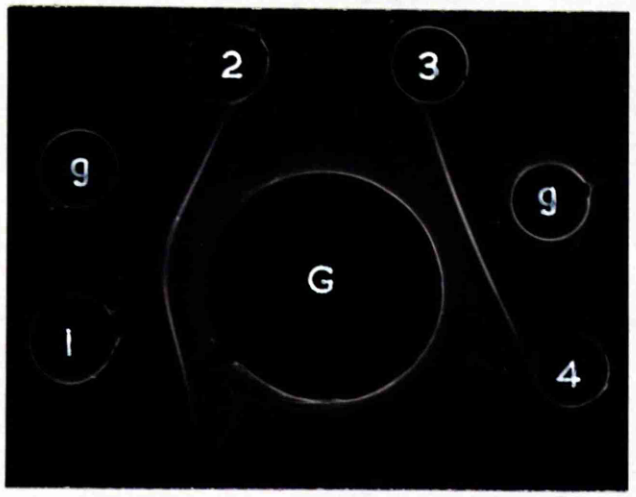
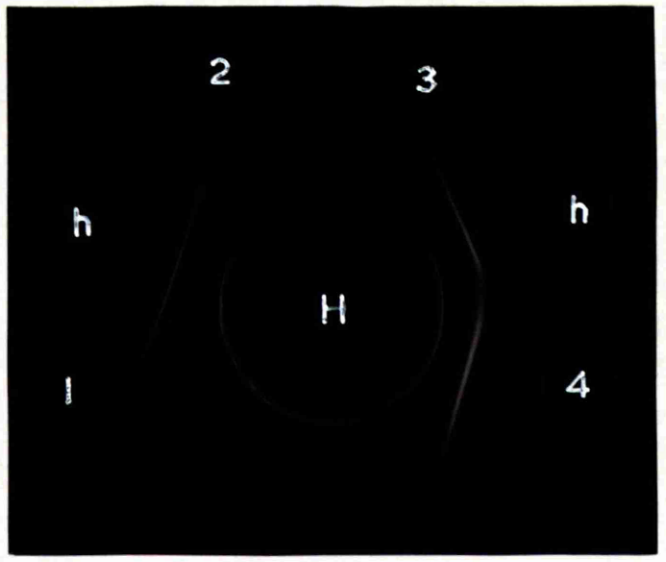
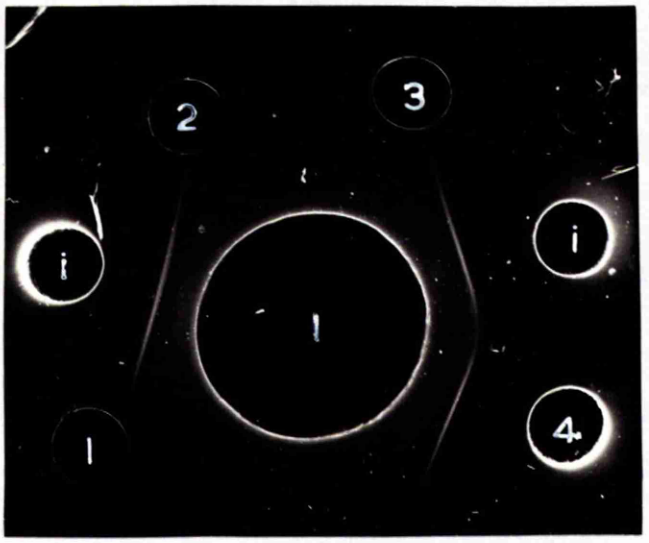
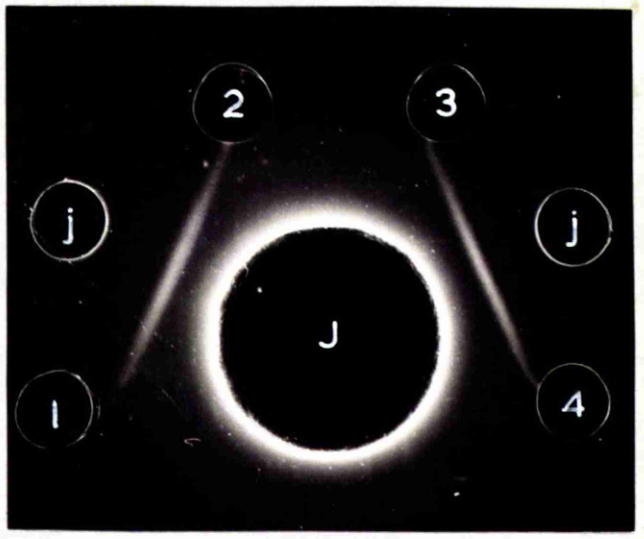


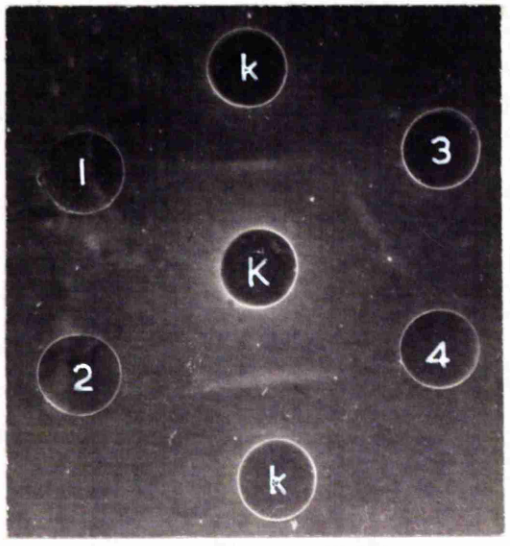
PLATE 5 (Contd.)







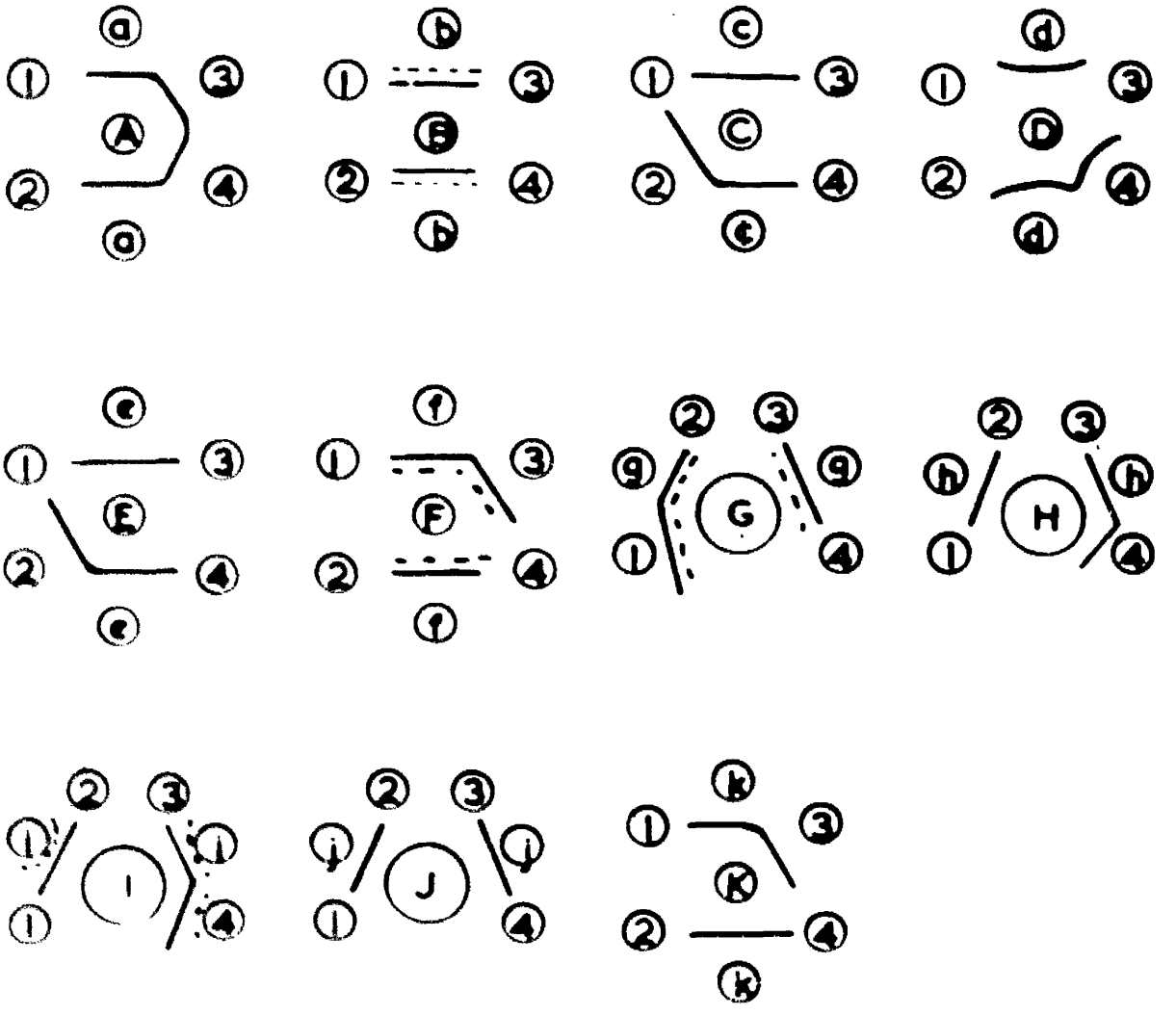




a-k - control antigens
A-K - antisera

Test crystal solutions:-
1 - Morrison g
2 - PIL 89 ce
3 - P 5 afk
4 - Steinhaus 1715 adhi

FIGURE 9 DIAGRAMMATIC ILLUSTRATION OF REACTIONS OF CRYSTAL SOLUTIONS OF 4 ISOLATES WITH THE 11 DIFFERENT ANTISERA



a–k CONTROL ANTIGENS

A–K ANTISERA

TEST CRYSTAL SOLUTIONS :–

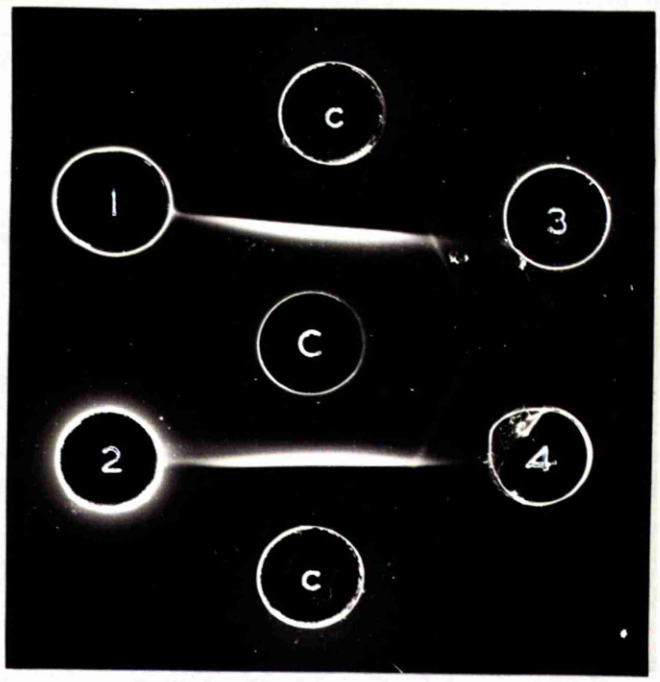
1–MORRISON g

2–PIL 89 ce

3–PS afk

4–STEINHAUS 1715 adhi

PLATE 6 THE PRESENCE OF ANTIBODY E IN ANTISERUM C PREPARED AGAINST T 84-A CRYSTALS



c - solution of T 84-A crystals

C - antiserum to T 84-A crystals

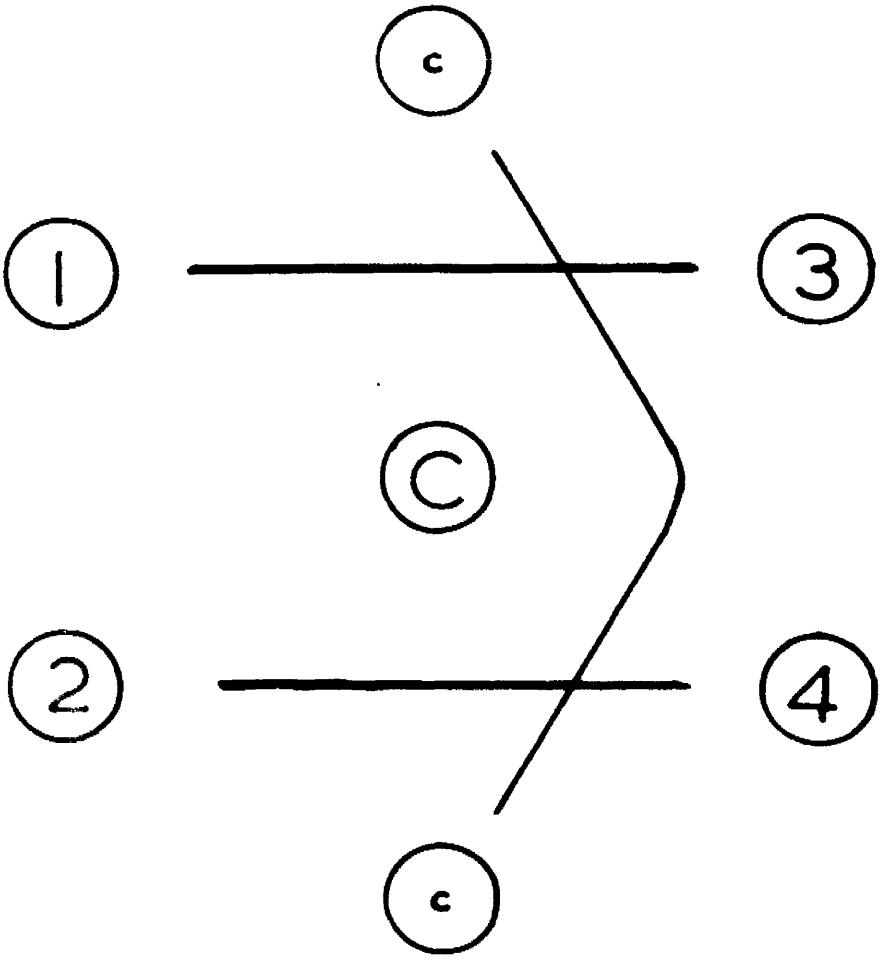
1 - solution of berliner crystals

2 - solution of Beira crystals

3 - solution of Ashman crystals

4 - solution of Ashton Rd. crystals

FIGURE 10 THE PRESENCE OF ANTIBODY E IN ANTISERUM C PREPARED AGAINST T84-A CRYSTALS



c — SOLUTION OF T84-A CRYSTALS

C — ANTISERUM TO T84-A CRYSTALS

1 — SOLUTION OF BERLINER CRYSTALS

2 — SOLUTION OF BEIRA CRYSTALS

3 — SOLUTION OF ASHMAN CRYSTALS

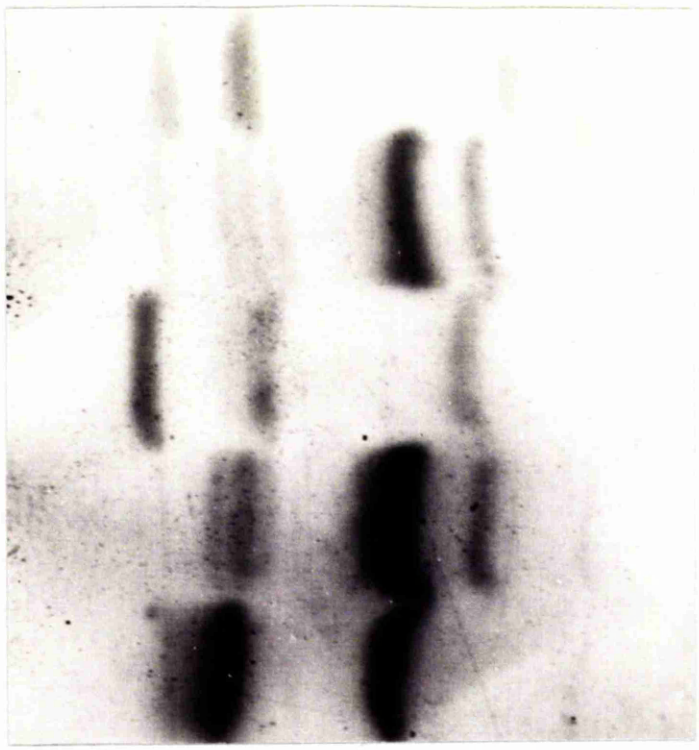
4 — SOLUTION OF ASHTON RD. CRYSTALS

reactions shown in fig.8 and Plate 4 where the precipitin line between antiserum and test antigen does not cross the control reaction. The significance of both types of reaction will be discussed later.

(c) Antigenic patterns of solutions of crystals of 94 isolates of B. thuringiensis

Vegetative cell disintegrates of these ninety-four isolates were prepared and examined for esterase pattern as described on p.52 . The isolates were classified on the basis of their esterase patterns using the nomenclature proposed by Norris - (1964). Many of the esterase types were checked by Dr. Norris of 'Shell Research Ltd.' (personal communication). Plate 7 shows the ten different esterase patterns. The table on the following page lists the ninety-four isolates, their esterase types, the antigens detected in soluble extracts of crystals and their crystal serotype. Plate 4 and fig.9 show the gel diffusion patterns obtained with four of the ninety-four isolates when diffused against the eleven different antisera. Space does not permit plates illustrating all ninety-four isolates diffused against the eleven antisera but the protocol of most of the gel diffusion patterns is available on request.

PLATE 7 ELECTROPHORETIC ESTERASE PATTERNS OF VEGETATIVE CELL DISINTEGRATES OF VARIETIES OF B. THURINGIENSIS



- Tolworth
- Dendrolimus
- Entomocidus
- Galleriae
- Kenya

- Berliner
- Entomocidus
- Berliner
- Sotto
- Berliner

← Electrophoresis

Table 12

Antigens detected in solutions of crystals of 94 isolates of B. thuringiensis and their esterase groupings

ISOLATE STRAINS	ESTERASE GROUP	CRYSTAL ANTIGENS a	b	c	d	e	f	g	h	i	j	k	CRYSTAL SEROTYPE
AC 58	berliner	-	-	-	+	+	-	-	-	+	-	-	dei
AC 100	berliner	-	-	-	+	-	-	-	-	+	-	-	di
AC 112	berliner	+	-	-	+	-	-	-	-	+	-	-	adi
Bakthane	berliner	+	-	-	-	-	-	-	-	-	-	-	a
berliner	berliner	+	-	-	+	-	-	-	-	+	-	-	adi
Brunel	berliner	-	-	-	+	-	-	-	-	+	-	-	di
C.N.45	berliner	+	-	-	+	-	-	-	-	+	-	-	adi
C.N.220	berliner	-	-	-	+	-	-	-	-	+	-	-	di
C.N.221	berliner	+	-	-	+	-	-	-	-	+	-	-	adi
E 1	berliner	-	-	-	-	+	-	-	-	-	-	-	e
French Standard	berliner	+	-	-	+	-	-	-	-	+	-	-	adi
galleria allemand	berliner	-	-	-	-	-	-	-	-	+	-	-	i
Leeds/1	berliner	+	-	+	-	+	-	-	-	+	-	-	acei
Leeds/2	berliner	+	-	-	+	+	-	-	-	+	-	-	adei
Leith	berliner	+	-	-	+	-	-	-	-	+	-	-	adi
PIL 90	berliner	+	-	-	+	-	-	-	-	+	-	-	adi
Steinhaus 1715	berliner	+	-	-	+	-	-	-	+	+	-	-	adhi
thuringiensis BS	berliner	+	-	-	+	-	-	-	-	+	-	-	adi
thuringiensis LD	berliner	-	-	+	+	+	-	-	-	+	-	-	cdei
thuringiensis LDI	berliner	-	-	+	+	-	-	-	-	+	-	-	cdi
thuringiensis M	berliner	+	-	-	+	-	-	-	-	+	-	-	adi
thuringiensis Mattés	berliner	+	-	-	-	-	-	-	-	-	-	-	a
3624	berliner	+	-	-	+	-	-	-	-	+	-	-	adi
2 J	berliner	+	-	-	+	-	-	-	-	+	-	-	adi
2 H	berliner	-	-	-	+	-	-	-	+	+	-	+	dhik
2 L	berliner	+	-	-	+	-	-	-	-	+	-	-	adi
4 H	berliner	+	-	-	+	-	-	-	-	+	-	-	adi
15	berliner	-	-	-	+	-	-	-	+	+	-	-	dhi
4058/A	berliner	+	-	-	+	-	-	-	-	+	-	-	adi
91	berliner	-	-	-	-	-	+	-	+	+	-	-	fhi
finitimus	finitimus	-	+	-	-	-	-	-	-	-	-	-	b
anduze	alesti	+	-	-	+	-	-	-	-	-	-	-	ad
alesti	alesti	-	-	-	-	-	-	-	+	+	-	-	hi

Table 12 (contd.)

ISOLATE STRAINS	ESTERASE GROUP	CRYSTAL ANTIGENS a	b	c	d	e	f	g	h	i	j	k	CRYSTAL SEROTYPE
galleria euxoae	alesti	+	-	+	-	-	+	-	-	+	-	+	acfik
P 3	alesti	-	-	-	-	-	-	-	+	+	-	-	hi
P 4	alesti	-	-	-	-	-	-	-	+	+	-	-	hi
P 6	alesti	-	-	-	-	-	-	-	+	+	-	-	hi
4138	alesti	+	-	+	-	-	-	-	-	-	-	-	ac
77 MD 0559	alesti	-	-	-	-	-	-	-	+	+	-	-	hi
77 MD 0658	alesti	-	-	-	-	-	-	-	+	-	-	-	h
T 84-A	sotto	-	-	+	-	-	-	-	-	-	-	-	c
dendrolimus	dendrolimus	-	-	-	+	-	-	-	-	+	-	-	di
ACC 1716	kenya	-	-	-	-	+	-	-	+	-	-	-	eh
ACC 1731	kenya	-	-	-	-	+	-	-	-	-	-	-	e
Ashman	kenya	-	-	-	-	+	-	-	-	-	-	-	e
Ashton Rd.	kenya	-	-	-	-	+	-	-	-	-	-	-	e
Cardiff Rd.	kenya	-	-	-	-	+	-	-	-	-	-	-	e
Chicory	kenya	-	-	-	-	+	-	-	-	-	-	-	e
Eldoret	kenya	-	-	-	-	+	-	-	+	-	-	-	eh
Jacobs	kenya	-	-	-	-	+	-	-	-	-	-	-	e
Kenya	kenya	-	-	-	-	+	-	-	-	-	-	-	e
Nairobi	kenya	-	-	-	-	+	-	-	+	-	-	-	eh
PIL 94	kenya	-	-	-	-	+	-	-	-	-	-	-	e
Rhodesia	kenya	-	-	-	-	+	-	-	+	-	-	-	eh
Steinhaus 1748	kenya	-	-	+	-	+	-	-	-	-	-	-	ce
Beira	galleriae	-	-	+	-	-	-	-	-	-	+	-	cj
G 1	galleriae	-	-	-	-	-	+	-	+	-	-	+	fhk
galleriae russe	galleriae	-	-	-	-	-	+	-	+	-	-	+	fhk
HA-3	galleriae	-	-	+	-	-	-	-	-	-	-	-	c
Hill	galleriae	-	-	-	-	-	+	-	-	-	-	+	fk
Larva 106	galleriae	-	-	-	-	-	+	-	-	-	-	+	fk
PIL 89	galleriae	-	-	+	-	+	-	-	-	-	-	-	ce
PIL 93	galleriae	-	-	-	-	+	-	-	-	-	-	-	e
PIL 95	galleriae	-	-	+	-	+	-	-	-	-	-	-	ce
PIL 96	galleriae	-	-	+	-	-	-	-	-	-	-	-	c
PIL 105	galleriae	-	-	-	-	-	-	-	-	-	+	-	j
PIL 106	galleriae	-	-	-	-	-	+	-	-	-	-	-	f
PIL 122	galleriae	-	-	+	-	-	-	-	-	-	+	-	cj
plodia	galleriae	-	-	-	-	-	-	-	+	-	+	-	hj
plodia interpunctella	galleriae	-	-	-	-	-	-	-	-	-	+	-	j

Table 12 (contd.)

ISOLATE STRAINS	ESTERASE GROUP	CRYSTAL ANTIGENS a	b	c	d	e	f	g	h	i	j	k	CRYSTAL SEROTYPE
Pretoria	galleriae	-	-	+	-	+	-	-	-	-	-	-	ce
Schvetsova	galleriae	-	-	-	-	-	-	-	+	-	-	+	hk
T 120	galleriae	-	-	-	-	-	+	-	-	-	-	-	f
T 63L4	galleriae	-	-	-	-	-	-	-	+	-	+	-	hj
058R	galleriae	-	-	-	+	-	-	-	-	+	-	-	di
5841	galleriae	-	-	+	-	-	-	-	-	-	+	-	cj
1 H-A	galleriae	-	-	+	-	-	-	-	-	-	+	-	cj
2K	galleriae	-	-	-	-	-	-	-	+	-	-	+	hk
ACC 1891	entomocidus	+	-	+	-	-	-	-	-	-	-	-	ac
entomocidus	entomocidus	+	-	+	-	-	-	-	-	-	+	-	acj
Limassol	entomocidus	+	-	+	-	-	-	-	-	-	-	-	ac
subtoxicus	entomocidus	+	-	-	-	-	-	-	-	-	-	-	a
3625	entomocidus	+	-	-	-	-	-	-	-	-	-	-	a
3626	entomocidus	+	-	-	-	-	-	-	-	-	-	-	a
G 2	Morrison	-	-	-	-	-	-	+	-	+	-	-	gi
Morrison	Morrison	-	-	-	-	-	-	+	-	-	-	-	g
92	Morrison	-	-	-	+	-	-	+	-	+	-	-	dgi
Tolworth	Tolworth	-	-	-	-	+	-	-	-	-	-	-	e
Kilmarnock	Untyped	-	-	-	-	-	-	-	-	-	-	+	k
Slough	Untyped	-	-	+	-	-	-	-	-	-	+	-	cj
P 1	Untyped (alesti?)	-	-	-	-	-	-	-	-	-	-	+	k
P 2	Untyped (alesti?)	+	-	-	+	-	-	-	-	+	-	-	adi
826	B. cereus	+	-	-	+	-	-	-	-	+	-	-	adi
P 5	Untyped (alesti?)	+	-	-	-	-	+	-	-	-	-	+	afk

The results of the antigenic analysis are summarised on the following page with the isolate strains grouped under their esterase types for comparison.

(1) B. thuringiensis var. berliner

The 30 isolates of this esterase group contain in all 8 antigens which are combined to give 14 different serotypes. The most frequent serotype is adi which represents 14 of the 30 isolates. Furthermore, there is a strong association between the antigens d and i which are found together in 24 isolates; only 3 of the isolates have i without d. The isolates Bakthane and Mattés used commercially in the manufacture of insecticides share the serotype a.

(2) B. thuringiensis var. finitimus

The sole isolate contains a unique antigen b. This isolate is also unusual in that it is the only crystal-former which has the crystal attached to the spore.

(3) B. thuringiensis var. alesti

This group contains in all 7 antigens in 5 serotype combinations. The most frequent type is hi (5 out of 9 strains). The antigens d and i occur in this group but they are not associated to the same extent as they are in the berliner group. Six isolates contain i without d and one contains d without i. The isolates anduze and alesti, in the past considered very similar, now appear different in the antigenic pattern or serotype of their crystal solutions.

(4) B. thuringiensis var. sotto

The sole isolate investigated contains one antigen c which also occurs in combination with other antigens in 5 other

esterase groups especially the galleriae group.

(5) B. thuringiensis var. dendrolimus

The sole isolate contains the antigens d and i which occur together in several other esterase groups, i.e. berliner, galleriae and Morrison.

(6) B. thuringiensis var. kenya

The 13 isolates in this group contain 3 antigens, h, c and e. The e antigen is found in all isolates and the majority of isolates showed only antigen e, i.e. serotype e is the most frequent. The isolate Steinhaus 1748 has the serotype ce which occurs in the galleriae group also.

(7) B. thuringiensis var. galleriae

The 23 isolates in this large group have been divided into 2 sub-groups very similar in esterase patterns but distinguishable on the basis of the H antigen. The group is complex in so far as the crystal antigens are concerned and contains 8 of the 11 antigens in 10 combinations; the serotype cj is most frequent.

(8) B. thuringiensis var. entomocidus

All the 6 isolates investigated contain the antigen a. The Limassol isolate was separated by Norris - (1964) from the other entomocidus isolates since it gave a positive Voges-Proskauer reaction but it is serologically identical to the entomocidus isolate.

(9) B. thuringiensis var. Morrison

Although the 3 isolates of this group have different serotypes, they all share the antigen g which is not found in any other esterase group.

(10) B. thuringiensis var. Tolworth

The sole isolate has the serotype e which is associated with the kenya group in particular. There is also a strong similarity in biochemical reactions between the Tolworth and kenya esterase types although their esterase patterns are not similar (Norris - 1964).

Five isolates were not typed on the basis of esterase pattern (Norris, personal communication). The isolates P 1 and Kilmarnock appear to be similar - both are untyped for esterase pattern and share the serotype k. Isolates P 2 and 826 have serotypes adi which are typical of the berliner group. The isolate P 5 has the serotype af which does not occur in any esterase group described above.

The isolates Leeds/1 and Leeds/2, although differing in their crystal serotypes, were obtained by plating out an isolate labelled Leeds which presumably consisted of a mixture of strains.

3. Antigenic Analysis of Digests of Crystals

(a) Fractionation of digest

Antigenic analysis of crystal digests by the same procedure described on p.47 used for antigenic analysis of alkali extracts of crystals showed that the antigens present in crystal solutions did not always correspond to the antigens present in crystal digests.

The fractionation of crystal digests enabled two fractions - C1 and C2 to be distinguished. The former had a molecular weight greater than 200,000 and the latter had a molecular weight between 5,000 and 10,000. This will be described fully in the following section.

Preliminary antigenic analysis of fraction C1 from several strains showed that the fraction invariably contained the same antigens as were detected in alkali extracts of crystals. Antigenic analysis of fraction C2, however, showed that the antigenic pattern was frequently different from that of the crystal solutions. Also the toxicity of fraction C2 on injection into larvae of Pieris brassicae (p. 92) and its low molecular weight made it particularly interesting.

On these basis it was decided to analyse fraction C2 of the digests of crystals of the 94 isolates of crystal-formers. As described on p.45 it was possible to prepare

fraction C2, without column chromatography, by a secondary digestion so that fraction C1 was removed from the digest.

(b) Antigenic patterns of digests of crystals of 94 isolates of B. thuringiensis

The method is described in full on p. 47. The antigenic patterns of fraction C2 of 93 of the 94 isolates of B. thuringiensis are given below and were determined by the same method used to analyse the antigens in alkali extracts of crystals. One isolate, PIL 93, became acrystalliferous and so it was not possible to determine the antigens of fraction C2.

Table 13

Antigens detected in fraction C2 of digests of crystals of 93 isolates of B. thuringiensis

ISOLATE STRAINS	ESTERASE TYPE	CRYSTAL SEROTYPE	FRACTION C2 ANTIGENS a	b	c	d	e	f	g	h	i	j	k	FRACTION C2 PATTERN
AC 58	berliner	dei	-	-	-	-	-	-	-	-	+	-	-	i
AC 100	berliner	di	+	-	-	-	-	-	-	+	+	-	-	ahi
AC 112	berliner	adi	+	-	-	-	-	-	-	+	+	-	-	ahi
Bakthane	berliner	a	+	-	-	-	-	-	-	-	+	-	-	ai
berliner	berliner	adi	+	-	-	-	-	-	-	+	+	-	-	ahi
Brunel	berliner	di	-	-	-	-	-	-	-	+	+	-	-	hi
C.N. 45	berliner	adi	+	-	-	-	-	-	-	+	-	-	-	ah
C.N. 220	berliner	di	+	-	-	-	-	-	-	+	+	-	-	ahi
C.N. 221	berliner	adi	+	-	-	-	-	-	-	+	-	-	-	ah
E 1	berliner	e	+	-	-	-	-	-	-	+	+	-	-	ahi
French Standard	berliner	adi	+	-	-	-	-	-	-	+	+	-	-	ahi
galleria allemand	berliner	i	-	-	-	-	-	-	-	-	+	-	-	i
Leeds/1	berliner	acei	+	-	-	-	-	-	-	+	+	-	-	ahi
Leeds/2	berliner	adei	+	-	-	-	-	-	-	-	-	-	-	a
Leith	berliner	adi	+	-	-	-	-	-	-	+	+	-	-	ahi
PIL 90	berliner	adi	+	-	-	-	-	-	-	+	+	-	-	ahi
Steinhaus 1715	berliner	adhi	+	-	-	-	-	-	-	+	+	-	-	ahi
thuringiensis BS	berliner	adi	+	-	-	-	-	-	-	-	+	-	-	ai
thuringiensis LD	berliner	cdei	-	-	-	-	-	-	-	-	-	-	-	-
thuringiensis LDI	berliner	cdi	-	-	-	-	-	-	-	-	+	-	-	i
thuringiensis M	berliner	adi	+	-	-	-	-	-	-	-	+	-	-	ai
thuringiensis Mattés	berliner	a	+	-	-	-	-	-	-	+	+	-	-	ahi

Table 13 (contd.)

ISOLATE STRAINS	ESTERASE TYPE	CRYSTAL SEROTYPE	FRACTION C2 ANTIGENS a	b	c	d	e	f	g	h	i	j	k	FRACTION C2 PATTERN
3624	berliner	adi	+	-	-	-	-	-	-	+	+	-	-	ahi
2 J	berliner	adi	+	-	-	-	-	-	-	+	+	-	-	ahi
2 H	berliner	dhik	-	-	-	-	-	-	-	-	+	-	-	i
2 L	berliner	adi	+	-	-	-	-	-	-	+	+	-	-	ahi
4 H	berliner	adi	+	-	-	-	-	-	-	+	-	-	-	ah
15	berliner	dhi	-	-	-	-	-	-	-	-	-	-	-	-
4058/A	berliner	adi	+	-	-	-	-	-	-	+	+	-	-	ahi
91	berliner	fhi	-	-	-	-	-	-	-	-	+	-	-	i
finitimus	finitimus	b	-	-	-	-	-	-	-	-	-	-	-	-
anduze	alesti	ad	+	-	-	-	-	-	-	+	+	-	-	ahi
alesti	alesti	hi	-	-	-	-	-	-	-	-	+	-	-	i
galleria euxoae	alesti	acfik	-	-	-	-	-	-	-	-	+	-	-	i
P 3	alesti	hi	-	-	-	-	-	-	-	-	-	-	-	-
P 4	alesti	hi	-	-	-	-	-	-	-	-	-	-	-	-
P 6	alesti	hi	-	-	-	-	-	-	-	-	-	-	-	-
4138	alesti	ac	-	-	-	-	-	-	-	-	-	-	-	-
77 MD 0559	alesti	hi	-	-	-	-	-	-	-	-	+	-	-	i
77 MD 0658	alesti	h	-	-	+	-	-	-	-	-	-	-	-	c
T 84-A	sotto	c	-	-	-	-	-	-	-	-	+	-	-	i
dendrolimus	dendrolimus	di	-	-	-	-	-	-	-	-	+	-	-	i
ACC 1716	kenya	eh	-	-	-	-	+	-	-	-	+	-	-	ei
ACC 1731	kenya	e	-	-	+	-	-	-	-	-	+	-	-	ci
Ashman	kenya	e	-	-	-	-	-	-	-	-	+	-	-	i
Ashton Rd.	kenya	e	-	-	+	-	-	-	-	-	+	-	-	ci
Cardiff Rd.	kenya	e	-	-	+	-	-	-	-	-	-	-	-	c
Chicory	kenya	e	-	-	-	-	-	-	-	-	+	-	-	i
Eldoret	kenya	eh	-	-	-	-	-	-	-	-	+	-	-	i
Jacobs	kenya	e	-	-	+	-	-	-	-	-	+	-	-	ci
Kenya	kenya	e	-	-	-	-	-	-	-	-	+	-	-	i
Nairobi	kenya	eh	-	-	-	-	-	-	-	-	+	-	-	i
PIL 94	kenya	e	-	-	-	-	-	-	-	-	+	-	-	i
Rhodesia	kenya	eh	-	-	+	-	-	-	-	-	+	-	-	ci
Steinhaus 1748	kenya	ce	-	-	-	-	+	-	-	-	-	-	-	e
Beira	galleriae	cj	-	-	-	-	-	-	-	-	+	-	-	i
G 1	galleriae	fhk	-	-	-	-	-	-	-	-	+	-	-	i
galleriae russe	galleriae	fhk	-	-	-	-	-	-	-	-	+	-	-	i
HA-3	galleriae	c	-	-	-	-	-	-	-	-	+	-	-	i
Hill	galleriae	fk	-	-	-	-	-	-	-	-	+	-	-	i
Larva 106	galleriae	fk	-	-	-	-	-	-	-	-	-	-	-	-
PIL 89	galleriae	ce	-	-	-	-	-	-	-	-	+	-	-	i
PIL 93	galleriae	e	not known											
PIL 95	galleriae	ce	-	-	-	-	-	-	-	-	+	-	-	i
PIL 96	galleriae	c	-	-	-	-	-	-	-	-	+	-	-	i

Table 13 (contd.)

ISOLATE STRAINS	ESTERASE TYPE	CRYSTAL SEROTYPE	FRACTION C2 ANTIGENS a b c d e f g h i j k	FRACTION C2 PATTERN
PIL 105	galleriae	j	- - - - - - - - + - -	i
PIL 106	galleriae	f	- - - - - - - - + - -	i
PIL 122	galleriae	cj	- - - - - - - - + - -	i
plodia	galleriae	hj	- - - - - - - - + - -	i
plodia interpunctella	galleriae	j	- - - - - - - - + - -	i
Pretoria	galleriae	ce	- - - - - - - - + - -	i
Schvetsova	galleriae	hk	- - - - - - - - - - -	-
T 120	galleriae	f	- - - - - - - - - - -	-
T 63L4	galleriae	hj	- - - - - - - - + - -	i
058R	galleriae	di	- - - - - - - - + - -	i
5841	galleriae	cj	- - - - - - - - + - -	i
1 H-A	galleriae	cj	- - - - - - - - + - -	i
2K	galleriae	hk	- - - - - - - + + - -	hi
ACC 1891	entomocidus	ac	+ - - - - - - + + - -	ahi
entomocidus	entomocidus	acj	+ - - - - - - + + + -	ahij
Limassol	entomocidus	ac	+ - - - - - - + + - -	ahi
subtoxicus	entomocidus	a	+ - - - - - - + - - -	ah
3625	entomocidus	a	+ - - - - - - + - - -	ah
3626	entomocidus	a	+ - - - - - - + - - -	ah
G 2	Morrison	gi	- - - - - - + - - - -	g
Morrison	Morrison	g	- - - - - - + - - - -	g
92	Morrison	dgi	- - - - - - + - + - -	gi
Tolworth	Tolworth	e	- - - - - - - - + - -	i
Kilmarnock	Untyped	k	- - - - - - - - + - -	i
Slough	Untyped	cj	- - - - - - - - + - -	i
P 1	Untyped (alesti?)	k	- - - - - - - - + - -	i
P 2	Untyped (alesti?)	adi	+ - - - - - - + + - -	ahi
826	B. cereus type	adi	+ - - - - - - + + - -	ahi
P 5	Untyped (alesti?)	afk	- - - - - - - - - - -	-

(c) General summary of results

The results of the antigenic analysis can be summarised as follows:-

(1) 4 of the 11 antigens (b, d, f and k) were not detected in fraction C2 of any digest.

(2) Some antigens appear to resist digestion more than others, e.g. antigen a is found in fraction C2 in 28 out of the 31 isolates whose crystal solutions contain the antigen a. At the other extreme, antigen e is found in fraction C2 in 2 out of the 20 isolates whose crystal solutions contain the antigen e. The antigen g is the only antigen which is always found in fraction C2 of crystals containing this antigen.

(3) Although 36 different antigenic patterns or serotypes of crystals were distinguished in the 94 isolates on the basis of antigens in crystal solutions, only 14 different antigenic patterns of fraction C2 were distinguished in the same isolates. That is, several different crystal serotypes may give rise to the same antigenic pattern on digestion, e.g. isolate Bakthane (crystal serotype a) and isolate thuringiensis BS (crystal serotype adi) are digested to give the antigenic pattern ai in fraction C2.

(4) Furthermore, some serotypes may give rise to a diversity of fraction C2 patterns on digestion. For example, the crystal serotype adi found in 16 isolates is digested diversely to give:- ahi (fraction C2 of 11

of these isolates), ah (fraction C2 of 3 of these isolates) and ai (fraction C2 of 2 of these isolates). The following table expands this point by listing the diversity in fraction C2 antigenic patterns which may arise from crystal serotypes.

Table 14

Diversity of fraction C2 antigenic patterns of digests of crystals of identical serotypes

CRYSTAL SEROTYPE	NO. OF ISOLATES	FRACTION C2 ANTIGENIC PATTERN
a	3	ah
a	1	ahi
a	1	ai
ac	2	ahi
ac	1	-
acei	1	ahi
acfik	1	i
acj	1	i
ad	1	ahi
adei	1	a
adhi	1	ahi
adi	3	ah
adi	11	ahi
adi	2	ai
afk	1	-
b	1	-
c	3	i
cdei	1	-
cdi	1	-
ce	1	e
ce	3	i
cj	5	i
dei	1	i
dgi	1	gi
dhi	1	-
dhik	1	i
di	1	ahi
di	1	hi
di	2	i
di	1	-
e	1	ahi
e	1	c

Table 14 (contd.)

CRYSTAL SEROTYPE	NO. OF ISOLATES	FRACTION C2 ANTIGENIC PATTERN
e	3	ci
e	5	i
e	1	not known
eh	1	ei
eh	1	ci
eh	2	i
f	1	i
f	1	-
fhi	1	i
fhk	2	i
fk	1	i
fk	1	-
g	1	g
gi	1	g
h	1	c
hi	2	i
hi	3	-
hj	2	i
hk	1	hi
hk	1	-
i	1	i
j	2	i
k	2	i

- = none of the 11 antigens were detectable with the available antisera in fraction C2.

4. Digestion of Alkali Extracts of Crystals by Larval Proteases

Protein was extracted from crystals of the berliner strain by alkali for digestion studies as described on p. 42 . Digestion was carried out for four hours at 30°C at a concentration of 1 ml. of gut juice/30-50 mg. protein.

(a) Fractionation of digest by column chromatography

Estimation of protein content of fractions was carried out by absorption measurements at 280 mμ as described on p. 46 . The digest of alkali extract was passed through a column of 'Sephadex' G-10 in M/100 phosphate buffer pH 8. It was found that all the material of the digest was excluded on this medium, i.e. there was very little material with molecular weight less than 700 as estimated by absorption at 280 mμ. Similar results were obtained with 'Sephadex' G-25 (fig.11) indicating that the material consisted of peptides or proteins of molecular weight greater than 5000. The medium G-50 was then used and it was possible to fractionate the digest into (1) an excluded fraction (molecular weight greater than 10,000) and (2) two fractions (molecular weight 5000-10,000). This separation is shown in fig.12. The excluded material (E1 molecular weight greater than 10,000) was passed through columns of G-100 and G-200. On column G-100, all of E1 was excluded indicating that there was no

FIGURE 11 FRACTIONATION OF DIGEST OF ALKALI EXTRACT OF CRYSTALS OF BERLINER STRAIN ON 'SEPHADEX' G-25

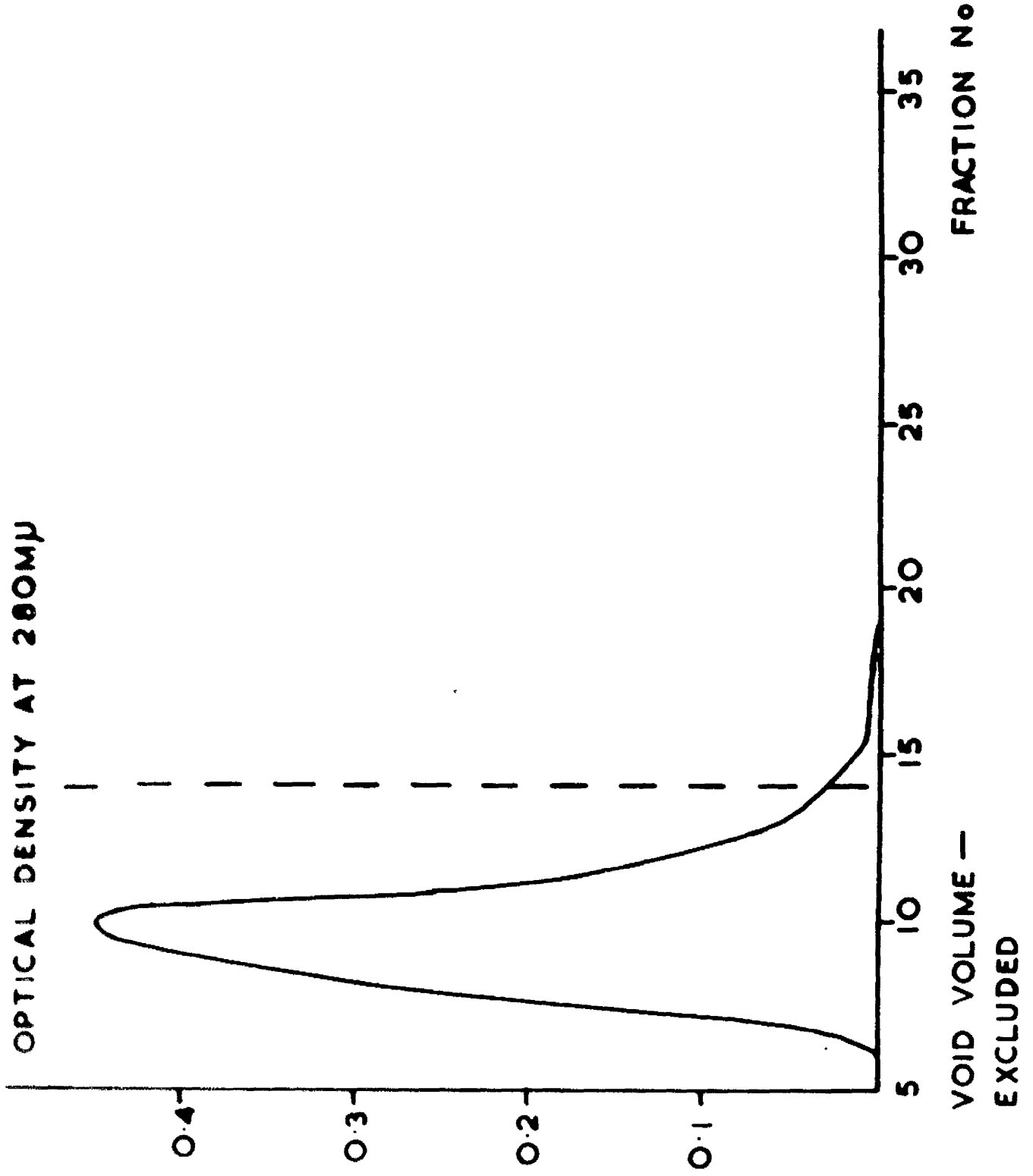
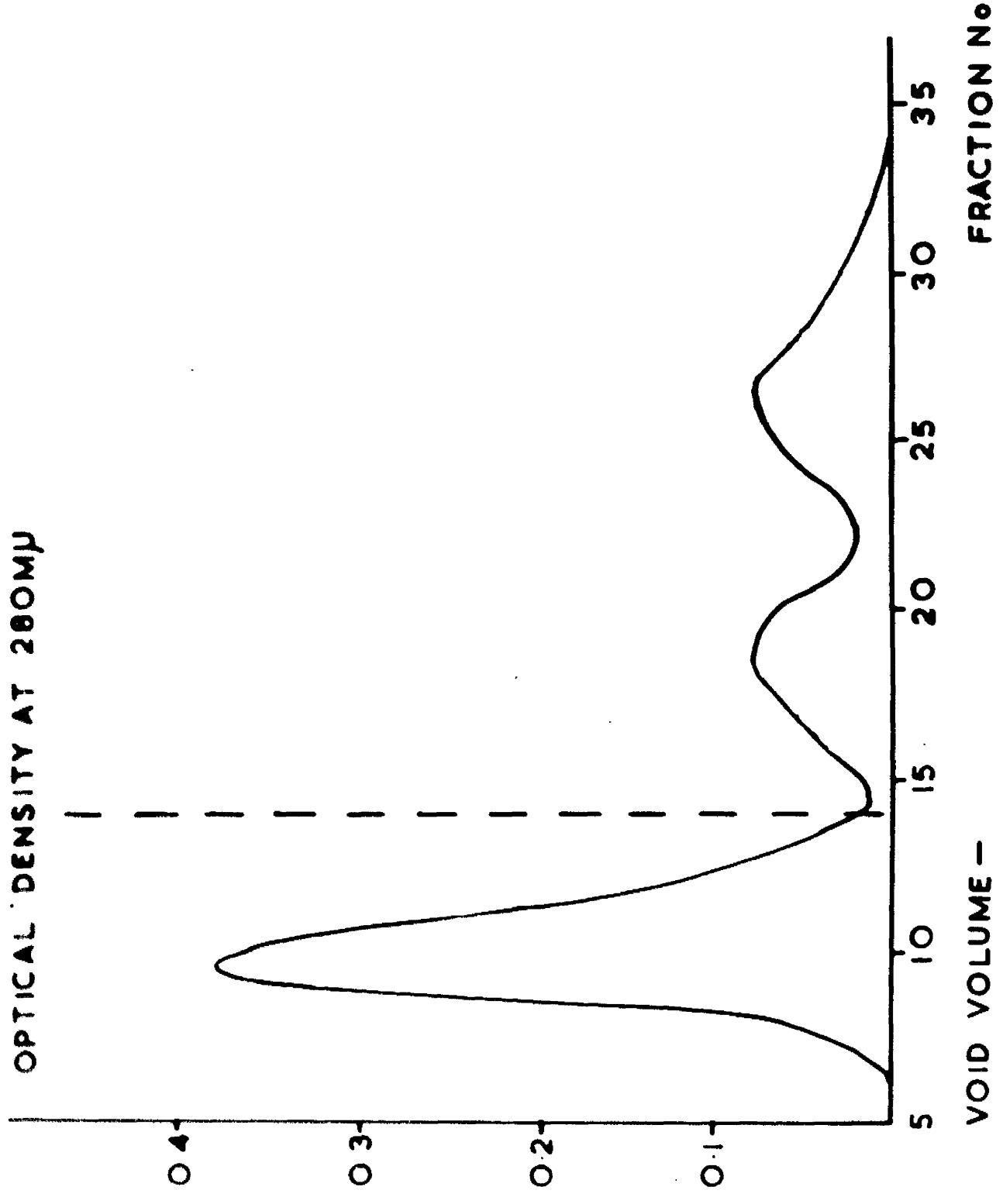


FIGURE 12 FRACTIONATION OF DIGEST OF ALKALI EXTRACTS OF CRYSTALS OF BERLINER STRAIN ON 'SEPHADEX' G-50



material in the digest of molecular weight between 10,000 and 100,000. When E1 was passed through G-200, the material was separated into a pattern of peaks similar to those obtained by passing the whole digest through G-50 and gave an excluded peak of molecular weight greater than 200,000 (E4) and two peaks (E5 and E6) of molecular weights between 100,000 and 200,000 as in fig.13. The complete flow diagram for separation on G-25, G-50, G-100 and G-200 is shown in fig.14. The estimated molecular weights of these fractions are, therefore, based on their exclusion on or inclusion in various grades of 'Sephadex'.

Table 15

Molecular weights of the 6 fractions of a digest of alkali extracts of berliner crystals

FRACTION	'SEPHADEX' GEL FILTRATION		MOLECULAR WEIGHTS
	EXCLUDED FROM	INCLUDED IN	
E1	G-50	separated on G-200	+ 10,000
E2	G-25	G-50	5,000-10,000
E3	G-25	G-50	5,000-10,000
E4	G-200	-	+200,000
E5	G-100	G-200	100,000-200,000
E6	G-100	G-200	100,000-200,000

(b) Toxicity of fractions of digest

Fractionation of the digested alkali extract of the crystals resulted in five distinct fractions - E2, E3, E4, E5 and E6 (E1 is equivalent to E4 + E5 + E6). The fractions were not

FIGURE 13 FRACTIONATION OF FRACTION E1 OF DIGEST OF ALKALI EXTRACTS OF CRYSTALS OF BERLINER STRAIN ON 'SEPHADEX' G—200

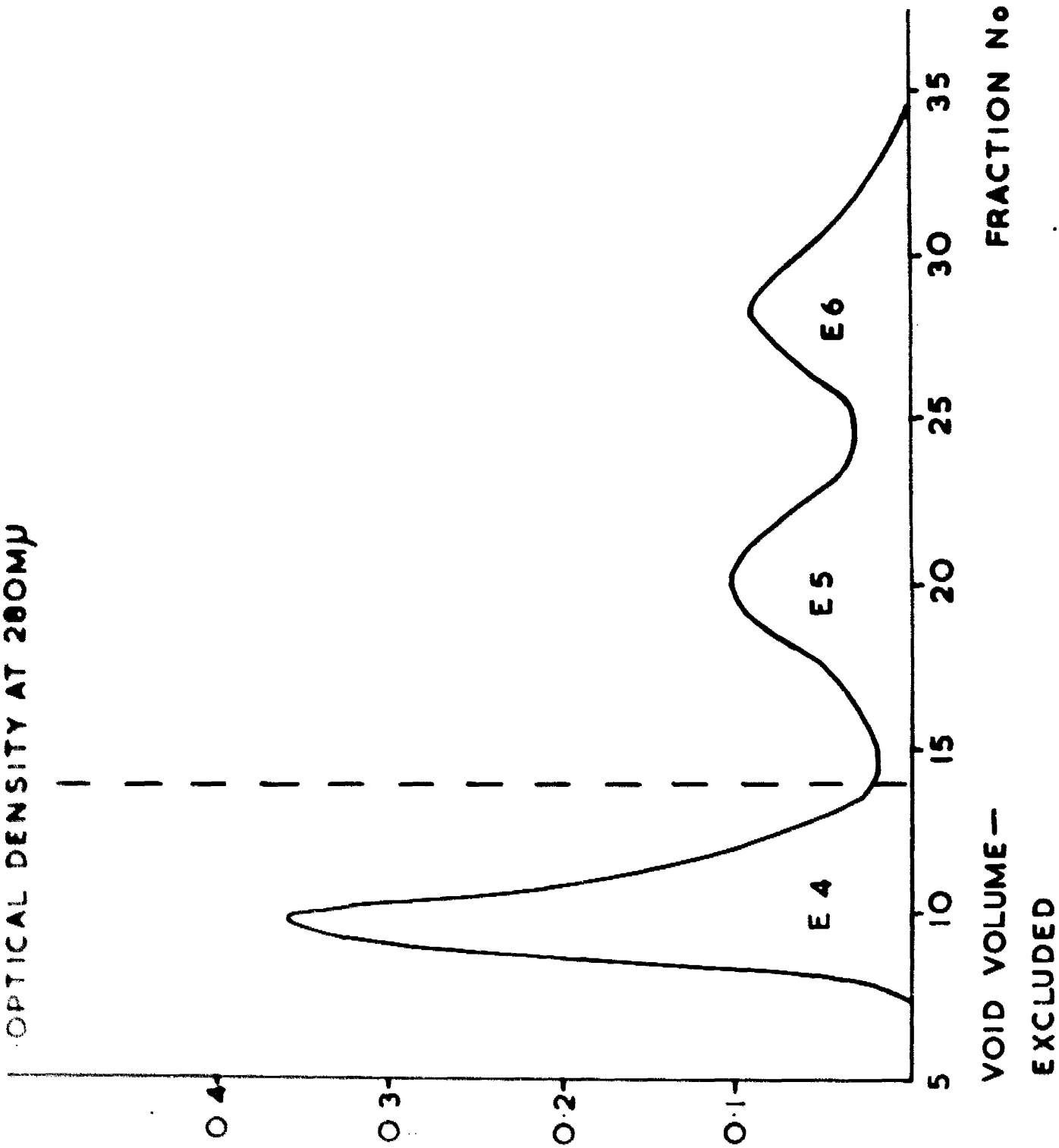
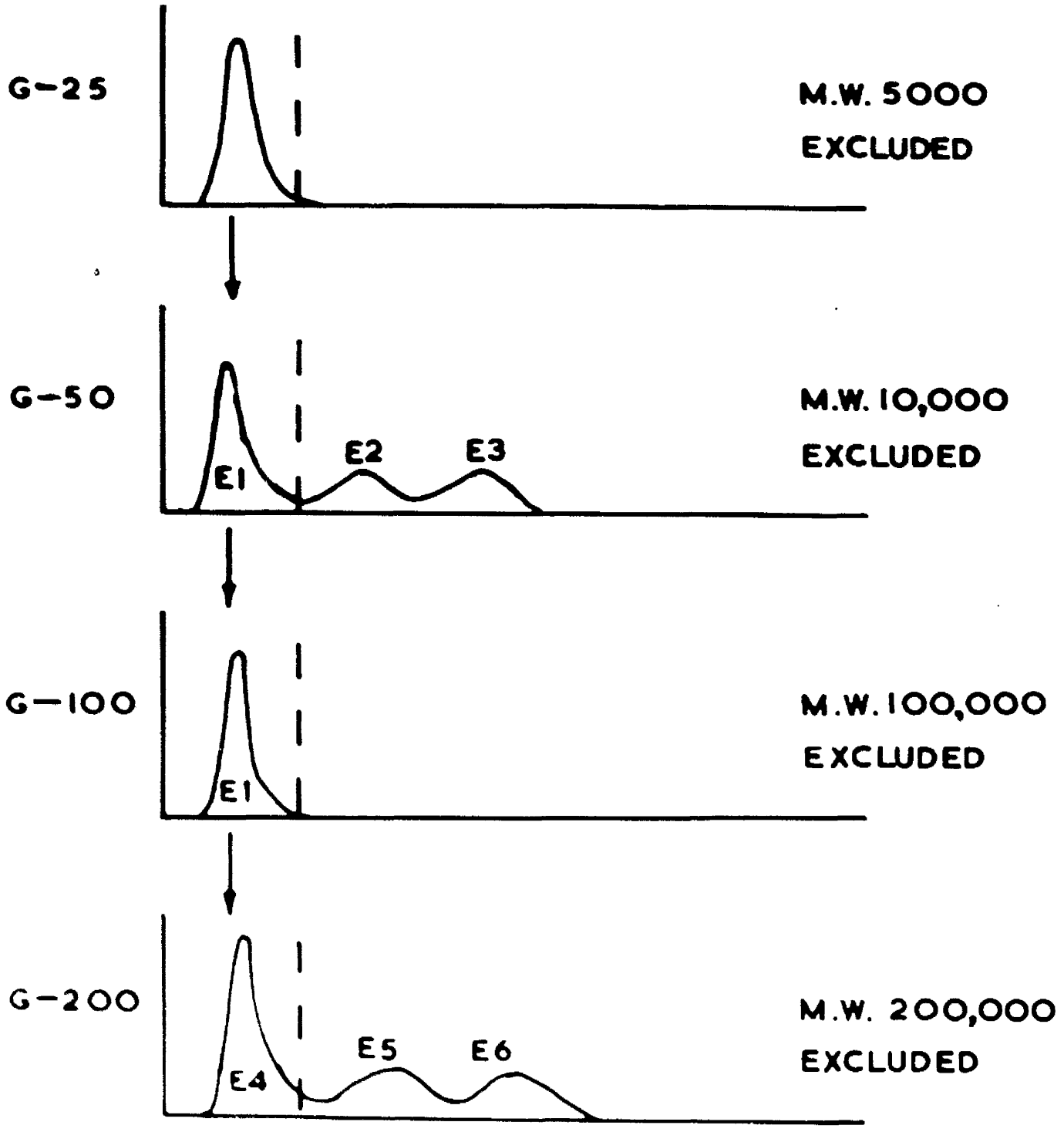


FIGURE 14 FLOW DIAGRAM — SEPARATION OF FRACTIONS E1–E6 ON VARIOUS GRADES OF SEPHADEX



always completely separated on the columns and accordingly fractions which corresponded to the mixture of material from two adjacent peaks were discarded. The fractions which were retained for further investigation are outlined in figs.12 and 13.

The fractions were concentrated by mixing with dry 'Sephadex' G-10 or G-25 gels which swelled and so absorbed solutes less than their respective exclusion limits. Since there was no peptide or protein in the digest with a molecular weight less than 5,000, only the phosphate buffer was absorbed by the gels, thereby concentrating the digest fractions. The samples were concentrated then sterilised by filtration and adjusted to 150 µg./ml. estimated from the standard curve described on p. 46 . The fractions were tested for toxicity as described on p.56 .

Table 16

Toxicity for larvae of Pieris brassicae of fractions of crystal extract digest

FRACTION	TOTAL NO. OF LARVAE DEAD AFTER DAYS INGESTION-(20 LARVAE) 1 DAY	2 DAYS	INJECTION-(50 LARVAE) 3 DAYS	7 DAYS
E1	18	20	9	15
E2	0	1	8	14
E3	0	0	3	7
E4	20	20	7	13
E5	1	1	4	8
E6	0	0	3	3
E2+E3+E5+E6	2	2	11	30
whole digest	19	20	6	22
crystal extract	20	20	not tested	not tested
phosphate buffer	0	0	4	6

From these results it was concluded that only material with a molecular weight over 200,000 was toxic by ingestion. The results of toxicity tests by injection are not conclusive in that several fractions (E1, E2 and E4) are apparently more toxic than the control phosphate buffer but are not as toxic as the whole (unfractionated) digest or as a mixture of the four lowest molecular weight fractions - E2, E3, E5 and E6.

(c) Significance tests on toxicity results

Since the control phosphate buffer had a moderate lethal effect of six dead larvae out of fifty, it was necessary to estimate the significance of the results of injecting larvae with test fractions. Although the results of ingestion experiments are more conclusive, significance tests were applied to these results also. The significance tests indicate how probable it was that the variation in numbers of deaths was due to chance or to real differences in toxicity. The following example shows how significance tests and values were calculated. The formulae are those of Moroney - (1953). This method involves the Null Hypothesis, that is, an assumption that there is no real significant difference between test and control samples. It is the degree of deviation from the expected standard error which determines significance.

Fraction E1 injection kills 15/50 larvae after 7 days.

Control phosphate buffer injection kills 6/50 larvae after 7 days.

The proportion of deaths for the combined sample is:-

$$p = \frac{\text{total deaths}}{\text{total tested}} = \frac{6 + 15}{50 + 50} = \frac{21}{100} = 0.21$$

The standard error of the difference in proportions between the two samples of $n_1 = 50$ and $n_2 = 50$ individuals will then be

$$\sigma_w = \sqrt{pq\left(\frac{1}{n_1} + \frac{1}{n_2}\right)} = \sqrt{0.21 \times 0.79\left(\frac{1}{50} + \frac{1}{50}\right)}$$

where p = probability $= \underline{0.0814}$

q = 1-p

The proportion of deaths in control group = 6/50 and the proportion of deaths in the test group = 15/50.

Hence the observed difference in proportions is $\frac{15}{50} - \frac{6}{50} = \underline{0.18}$

$$\frac{\text{Observed difference in proportions}}{\text{Standard error}} = \frac{0.18}{0.0814} = \underline{2.2}$$

Thus, in this example, the observed difference in proportions is equal to more than two standard errors. Moroney - (1953) explains that a difference of more than two standard errors is probably significant, i.e. these results could occur about once in 20 trials. According to Moroney - (1953), a figure of more that two represents a one in twenty chance occurrence. A figure of three is extremely significant and represents a chance of about one in 200. On this basis, values greater than two were regarded as significantly indicative of toxicity. The calculated significance values are shown in the table overleaf.

Table 17

Significance values of toxicity experiments with fractions of crystal extract digest

FRACTION	INGESTION	INJECTION
E1	9.1	2.2
E2	1.1	2.0
E3	0	0.3
E4	9.1	1.8
E5	1.1	0.1
E6	0	0
E2+E3+E5+E6	1.5	5.0
whole digest	9.1	3.6
crystal extract	9.1	not known

Lecadet & Martouret - (1962) described toxicity of crystal digests on injection at concentrations and doses similar to those reported above. One important difference between the method of Lecadet & Martouret and the method described is that Lecadet & Martouret prepared digests of crystals while the work reported in this section involved digestion of alkali extracts of crystals. The implications of these differences in methods and results of toxicity experiments will be discussed later.

It was decided to repeat the work of Lecadet & Martouret - (1962) by digesting crystals. The fractionation of this digest and the toxicity of the fractions are reported in the following section.

5. Digestion of Crystals by Larval Proteases

Crystals were prepared by methods described on p. 40 and were digested for three hours as described on p. 43 when the soluble products of digestion were investigated.

(a) Fractionation of digest by column chromatography

The digest of the crystals was passed through a column of 'Sephadex' G-10 equilibrated in M/100 phosphate buffer at pH 8. It was found that a small amount of the digest was present in the form of material of molecular weight less than 700 as estimated by its inclusion in the gel. Fig.15 shows the fractionation on G-10. The material of molecular weight greater than 700 was fractionated on 'Sephadex' G-50 (fig.16) into a fraction C1 which was excluded and so had a molecular weight greater than 10,000 and a fraction C2 which was included and so had a molecular weight of less than 10,000. C2 was excluded on the medium G-25, and C1 was excluded on the medium G-200 so that neither fraction was resolved further. The estimated molecular weights of C1 and C2 are given below.

Table 18

Estimated molecular weights of fractions of crystal digest

FRACTION	EXCLUDED ON	INCLUDED IN	MOLECULAR WEIGHT
C1	G-200	-	+200,000
C2	G-25	G-50	5,000-10,000

FIGURE 15 FRACTIONATION OF DIGEST OF BERLINER CRYSTALS ON SEPHADEX G-10

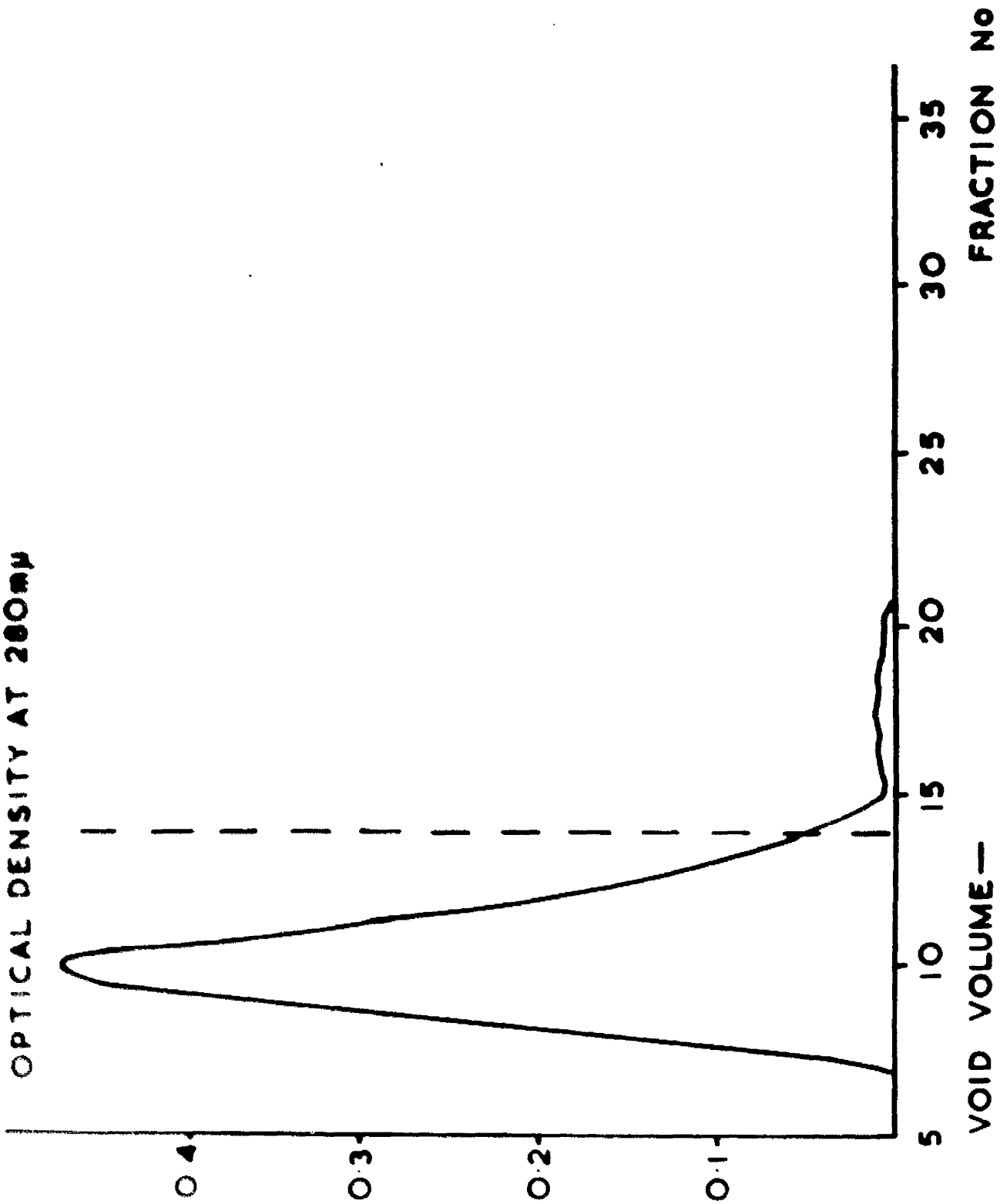
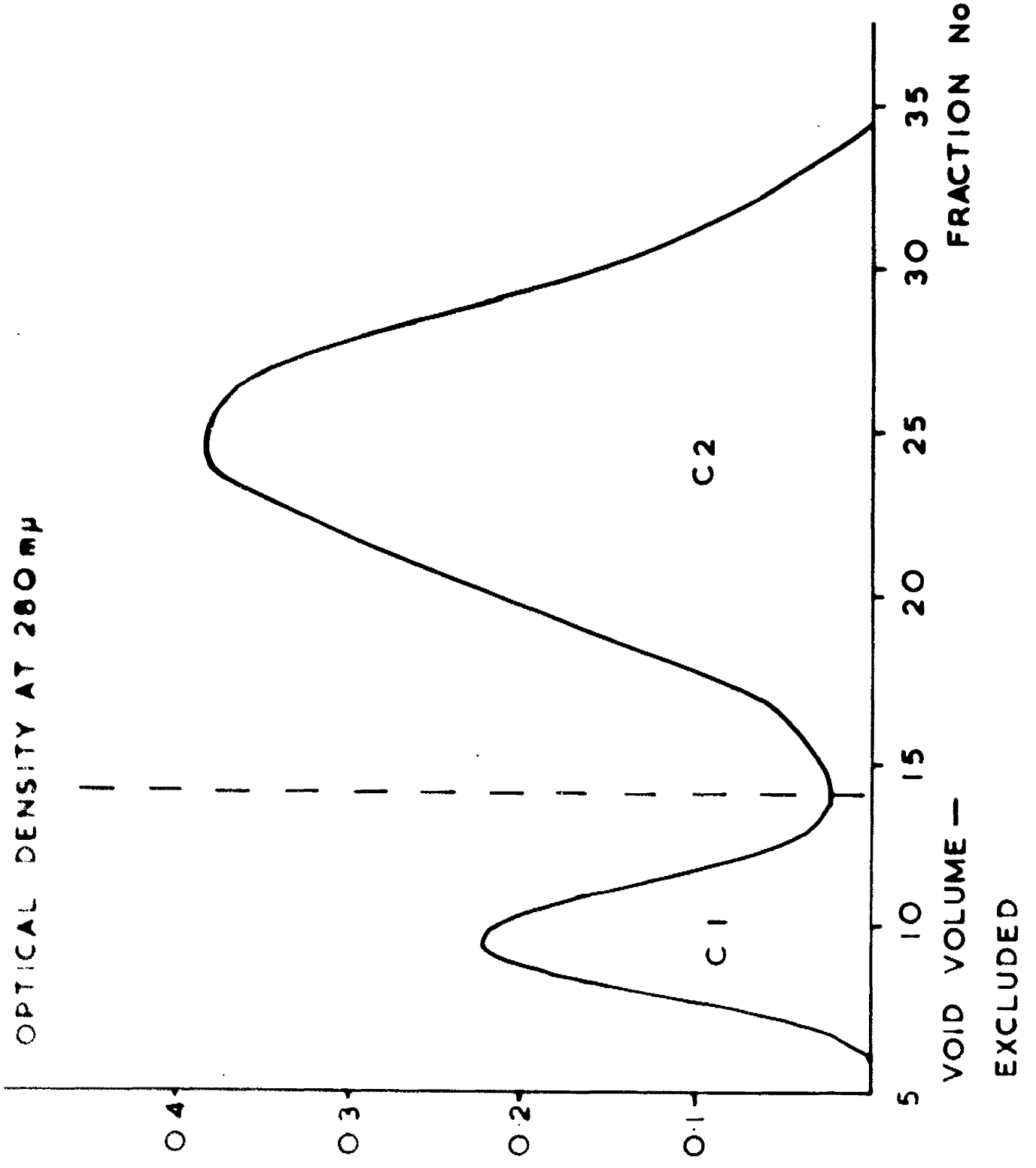


FIGURE 16 FRACTIONATION OF DIGEST OF BERLINER CRYSTALS ON SEPHADEX G-50



The results indicate that digestion of crystals gave rise to fewer digestion products than did digestion of crystal extracts. Digestion of crystals resulted in two fractions differing considerably in molecular weight. No material within the range 10,000-200,000 molecular weight was detected in the digests.

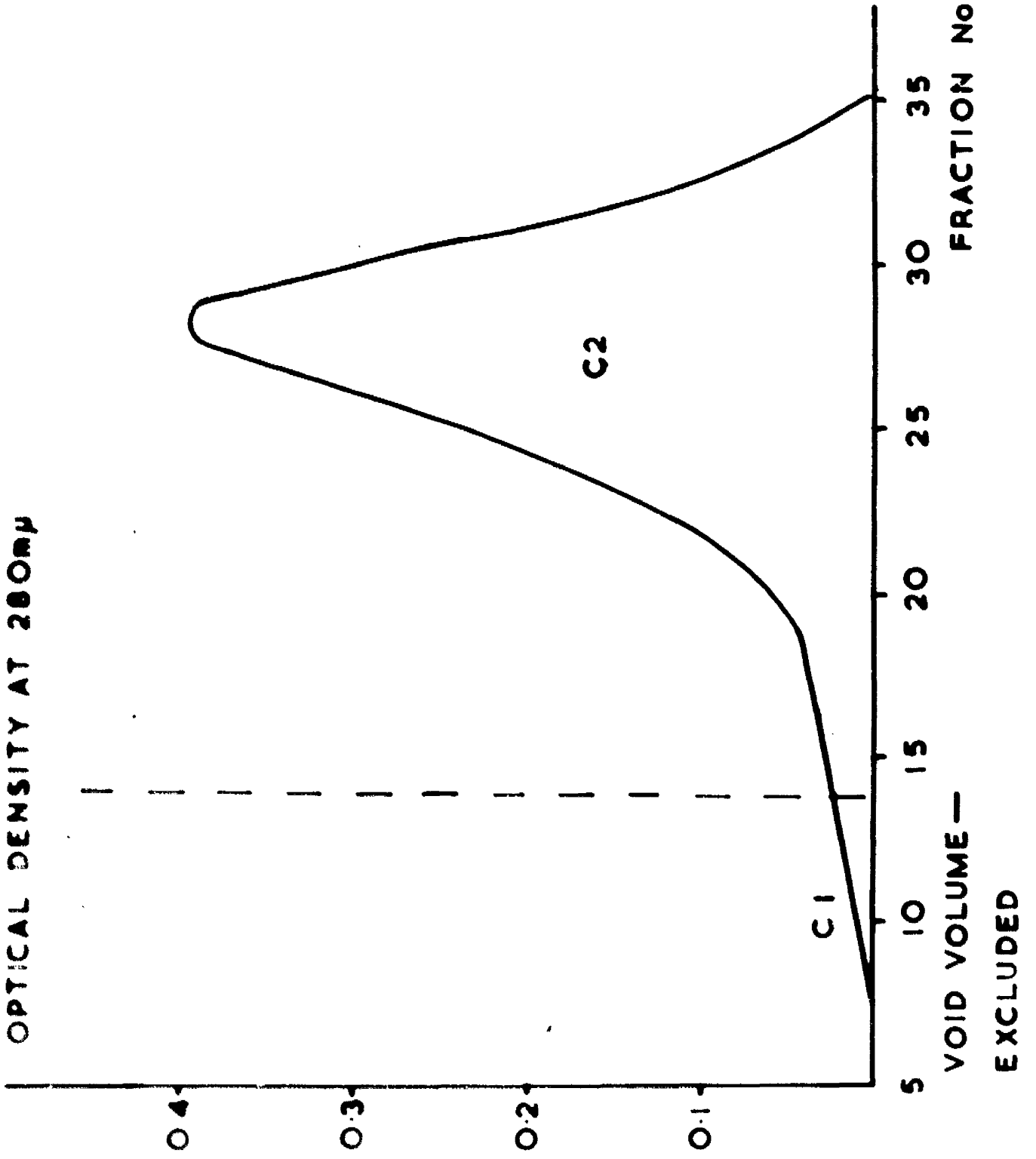
The crystal digests normally contained more of fraction C2 than C1 (fig.16). When the concentration of crystals in the digestion mixture was increased by about ten times, the proportion of fraction C1 increased so that approximately equal quantities of each fraction were present.

When a mixture of the two fractions was digested in the absence of crystals, it was found that after an hour C1 had disappeared and C2 had increased in amount. A comparison of the proportions of C1 and C2 before and after the secondary digestion is shown in fig.17. Similar results were obtained when the digest was incubated at $4^{\circ}C$ overnight. The results indicate that fraction C1 is an intermediate digestion product between the crystals and fraction C2.

(b) Toxicity of fractions of digest

The two fractions were precipitable with 50% ammonium sulphate and the small amount of material which was not precipitated had a molecular weight of less than 700 and was not toxic. Similarly, the whole digest (C1 and C2) was precipitable with 50% ammonium sulphate.

FIGURE 17 EFFECT OF SECONDARY DIGESTION OF CRYSTAL DIGEST ON FRACTION C1



The two fractions were concentrated by precipitation with 50% ammonium sulphate and re-dissolved in M/100 phosphate buffer at pH 8. Toxicity tests were carried out as described on p.56 . The results are given below:-

Table 19

Toxicity by injection and ingestion for larvae of Pieris brassicae of fractions of crystal digest

FRACTION	NUMBER OF LARVAE DEAD AFTER DAYS INGESTION (20 LARVAE) 1 DAY	2 DAYS	INJECTION (20 LARVAE) 3 DAYS	7 DAYS
C1	20	20	3	4
C2	20	20	12	18
whole digest	20	20	8	17
phosphate buffer	0	0	2	3

(c) Significance tests on toxicity results

The results in the table above were investigated by the same calculations as on p.86 of the 'Results' to determine significance values for toxicity results. These are given in the table below.

Table 20

Significance of toxicity experiments expressed by significance values

FRACTION	SIGNIFICANCE VALUE INGESTION	INJECTION
C1	9.1	0.41
C2	9.1	4.8
whole digest	9.1	4.4

These results show that both fractions are toxic by ingestion but only fraction C2 is toxic by injection. The smaller number of larvae tested in this experiment as compared with 50 larvae used in the previous section reflects the more conclusive results of the toxicity experiments of this section in contrast to the toxicity experiments on fractions of the crystal extract digest where several fractions are only moderately toxic. These results will be discussed later.

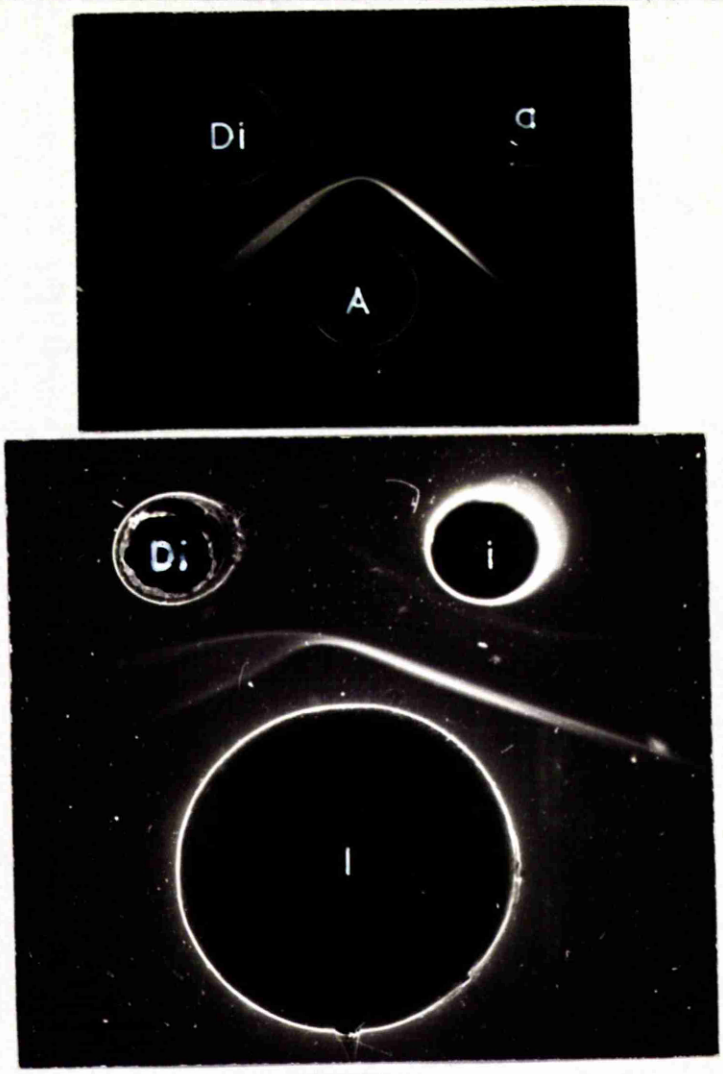
6. Immunoelectrophoresis and Gel Diffusion of Digests of Berliner Crystals

The results of the section 3 were investigated further in the case of the berliner strain with a view to estimating the number of antigenic determinants in fraction C1 and in fraction C2 and the number of peptides or proteins carrying the antigens in each fraction.

(a) Gel diffusion

Antigenic analysis of fraction C1 of the berliner crystal digest by gel diffusion showed that it contained the antigens a, d and i. Fraction C2 containedthe antigens a, h and i. Plate 8 and fig.18 show that the control antigen a gives a reaction of identity with the two precipitin lines formed by diffusion of the whole digest against antiserum A. FractionsC1 and C2 individually only give one precipitin line when diffused against antiserum A. Thus the two precipitin lines are due to two different molecules with different diffusion rates present in the whole digest, both of which carry the antigenic determinant a. Similar results were obtained when the whole digest was tested for antigen i. With antigens d and h which are present respectively in fractions C1 and C2, only one precipitin line was detected. The singularity of the precipitin lines in both fractions suggested that each antigenic determinant was present on one molecule only, although it was not possible to state whether

PLATE 8 GEL DIFFUSION PATTERNS OF UNFRACTIONATED CRYSTAL DIGEST DIFFUSED AGAINST ANTISERA A AND I



Di - unfractionated crystal digest

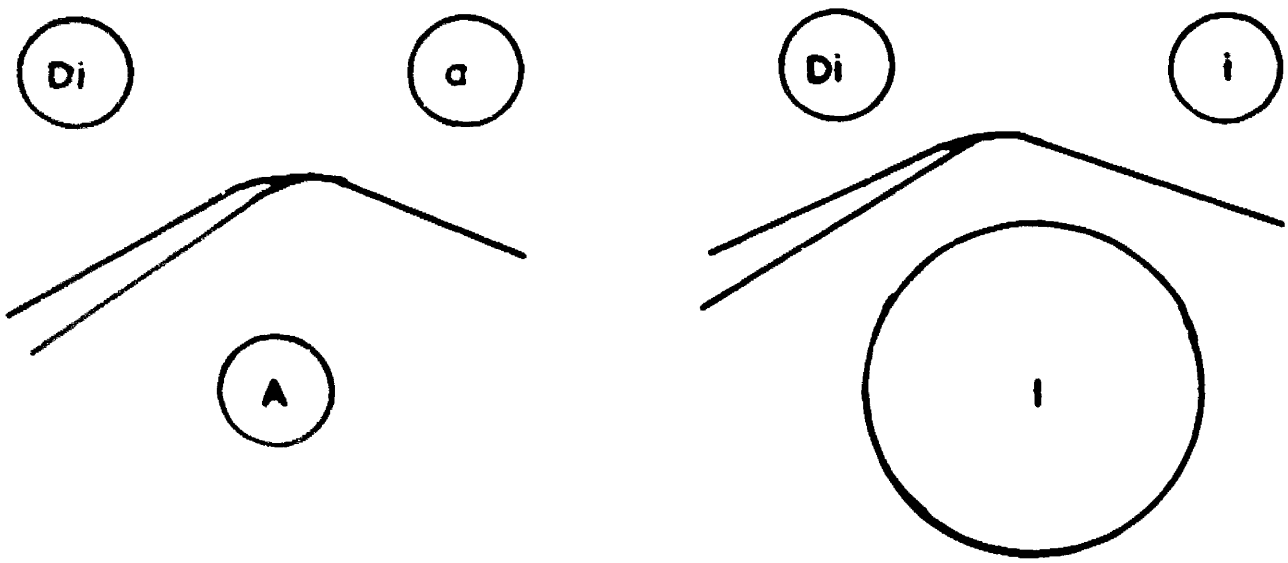
a - solution of berliner crystals

A - antiserum to berliner crystals

i - solution of galleria allemand crystals

I - antiserum to galleria allemand crystals

FIGURE 18 GEL DIFFUSION PATTERNS OF UNFRACTIONATED CRYSTAL DIGEST DIFFUSED AGAINST ANTISERA A AND I



DI — UNFRACTIONATED CRYSTAL DIGEST

a — SOLUTION OF BERLINER CRYSTALS

A — ANTISERUM TO BERLINER CRYSTALS

i — SOLUTION OF GALLERIA ALLEMAND CRYSTALS

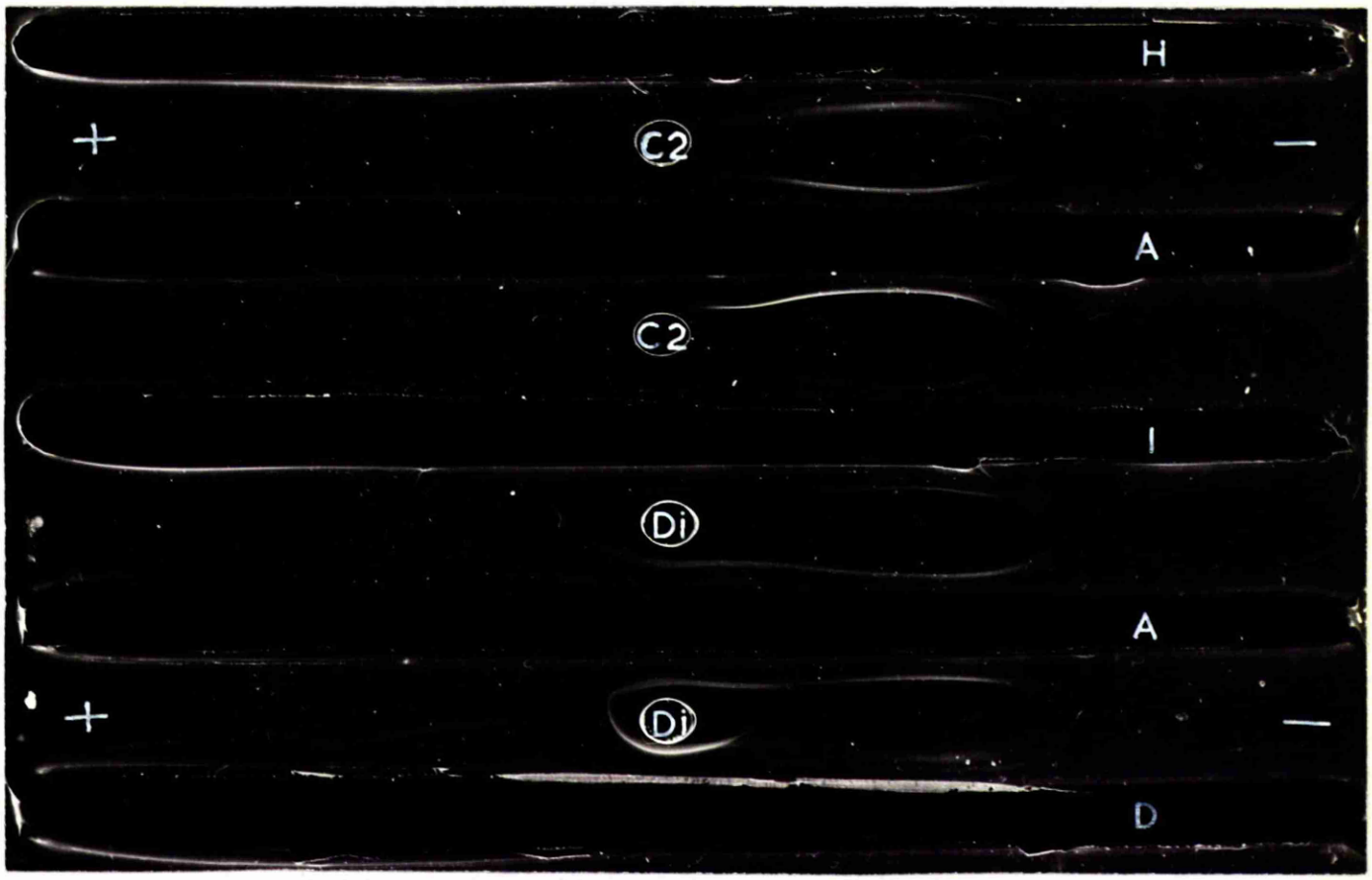
I — ANTISERUM TO GALLERIA ALLEMAND CRYSTALS

the three antigenic determinants a, h and i of fraction C2 were present on the same peptide or on separate peptide molecules.

(b) Immunoelectrophoresis

Gel immunoelectrophoresis combines the advantages of two separate methods (1) electrophoresis to separate the constituents of a mixture and (2) gel immunodiffusion to give precipitin lines or arcs by reaction of electrophoresed antigen mixture with antisera. The method is particularly applicable to complex antigen mixtures. Immunoelectrophoresis was applied to fractions C1 and C2 and to the whole digest as described on p. 48 . The stained gels of the digest against A, D, H and I antisera are shown in Plate 9 and fig.19. Fraction C1 gave a single precipitin arc with each of the antisera A, D and I and each of these arcs were situated at the original antigen well indicating that the material had not migrated in the electric field. The fact that antiserum D gave a reaction with material in this position but antiserum H does not indicates that this non-migrating material corresponds to fraction C1. Similarly, the other arc corresponds to fraction C2 since it reacts with antiserum H but not with D. This is confirmed by fraction C2 alone which reacts similarly and occupies a similar position in the gel. Fraction C2 migrated towards the cathode

PLATE 9 IMMUNOELECTROPHORESIS OF BERLINER CRYSTAL DIGEST WITH ANTISERA A, D, H AND I

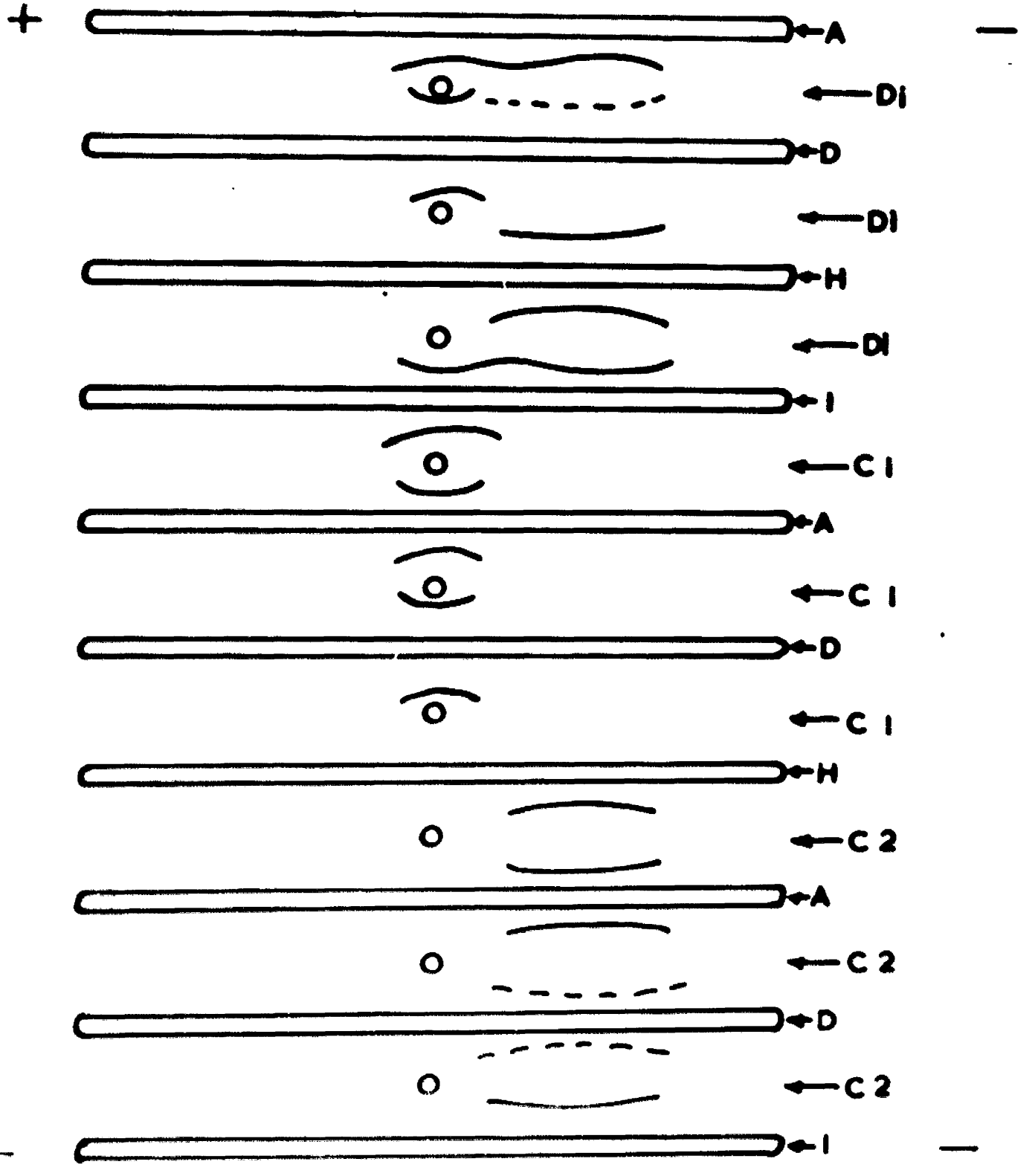


C2 - fraction C2 of crystal digest

Di - unfractionated berliner crystal digest

H, A, I, D - antisera

FIGURE 19 IMMUNOELECTROPHORESIS OF BERLINER CRYSTAL DIGEST AND ITS FRACTIONS C1 AND C2 WITH ANTISERA A, D, H AND I



Di - BERLINER CRYSTAL DIGEST

indicating that it consisted of positively charged ions.

These results are summarised in the following table:-

Table 21

Immunoelectrophoresis of digests of crystals of B. thuringiensis var. berliner

FRACTION	ANTIGENIC PATTERN	NO. OF PRECIPITIN ARCS	ELECTROPHORETIC MIGRATION
C1	adi	1	no migration
C2	ahi	1	towards cathode

These results strongly suggest that fraction C1 and fraction C2 each consist of a single protein or peptide.

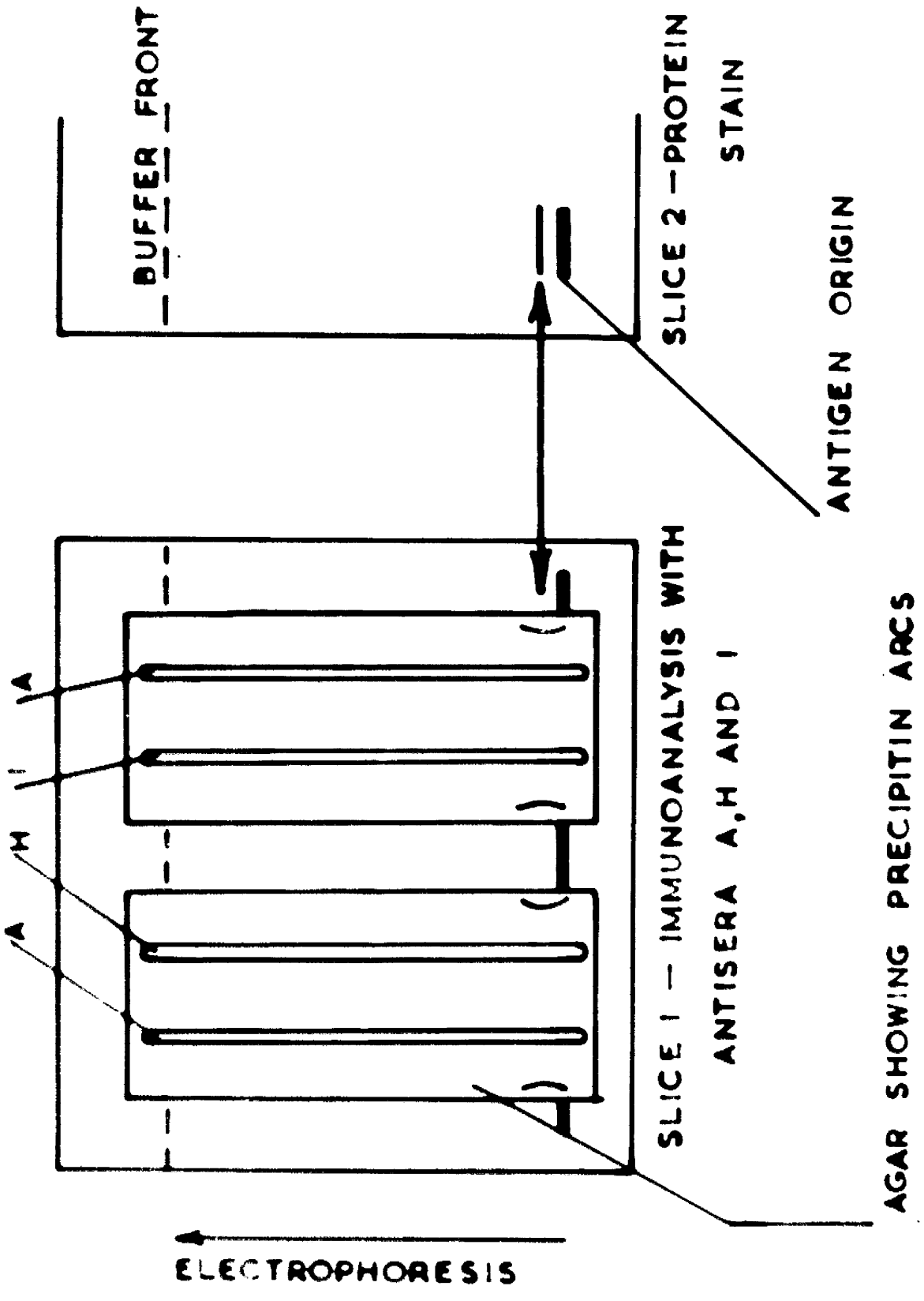
(c) Polyacrylamide gel immunoanalysis

This was carried out as described on p.48 and was applied to fraction C2. It was found that, with the normal discontinuous buffer system used in polyacrylamide gel, i.e. 'Tris' - citrate - borate as described on p. 48, fraction C2 did not enter the gel although it migrated towards the anodic side of the sample slit. Immunodiffusion with the antisera A, H and I resulted in a precipitin arc corresponding to the sample slit. When the polyacrylamide gel was formed in barbitone-acetate buffer instead of 'Tris' - citrate and the fraction electrophoresed as before, it was found that the antigenic material migrated a short distance into the gel towards the anode. The precipitin arcs obtained with the

antisera corresponded in position to a single strong protein band as was seen by staining another slice from the same gel with naphthalene black for protein staining. The E_f value of the fraction (0.05) was estimated from the ratio of distance travelled by the protein band to the distance travelled by the buffer front from the sample slit. The relationship between antigen arc and protein stain is shown in fig.20.

The results of these sections (a), (b) and (c) above give strong support to the possibility that the antigens in fraction C2 are present on the one peptide molecule. This concept will be discussed in more detail later.

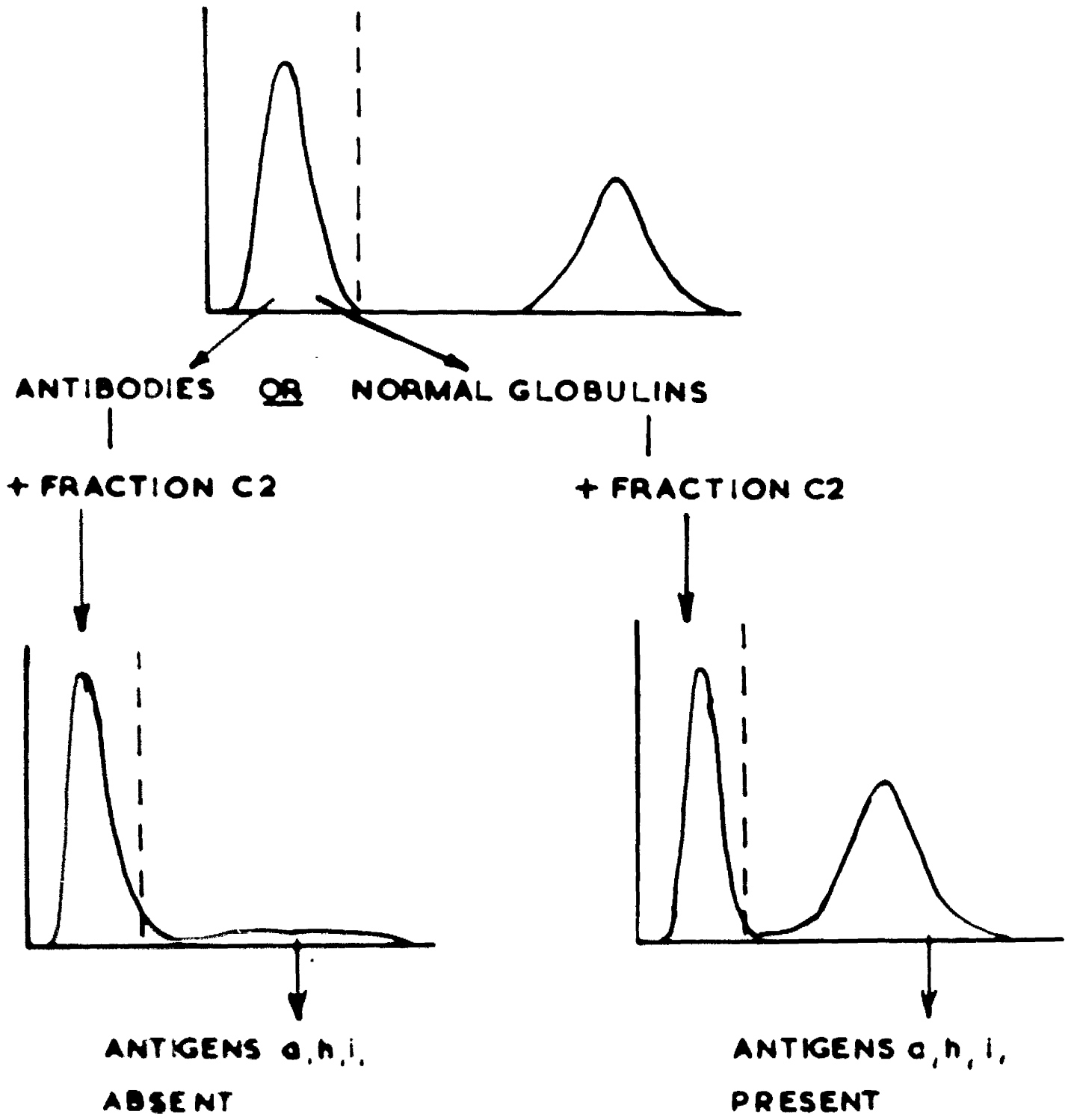
FIGURE 20 RELATIONSHIP BETWEEN ANTIGEN PRECIPITIN ARC AND PROTEIN BAND IN POLY-ACRYLAMIDE GEL IMMUNOANALYSIS OF FRACTION C 2



7. An Experiment to Determine Whether Antigens of Fraction C2 of the Crystal Digest were present on one or more Molecules

In this experiment use was made of the fact that the antibody molecules of the antisera A, H and I had molecular weights greater than 10,000 which is the exclusion limit of 'Sephadex' G-50, while the antigens in fraction C2 of the berliner crystal digest had a molecular weight of less than 10,000 and were consequently included in the same gel. Furthermore, antigen-antibody complexes of the antigens of fraction C2 with the antisera would also have a molecular weight greater than 10,000. Antigenic material which would not form part of this complex would be in the included fractions. The method is described on p. 51 .. It was found that after mixing with antiserum A less than 15% of fraction C2 remained in the included fractions of the gel. Fig.21 shows the difference in optical density between fraction C2 and fraction C2 mixed with antiserum A when passed through a column of 'Sephadex' G-50. The 15% of fraction C2 which remained was concentrated and tested by gel diffusion for the presence of antigens a, h, and i against antisera A, H and I with homologous antigens as controls. None of the antigens a, h and i appeared to be present in the material unabsorbed by the antiserum A. Similar results were obtained when the experiment was repeated with antiserum I. A control experiment with normal rabbit serum

FIGURE 21 FRACTIONATION OF FRACTION C2 ON SEPHADEX G-50 AFTER NEUTRALISATION WITH ANTISERA A AND I AND MIXTURE WITH NORMAL SERUM



showed that there was no absorption of fraction C2 indicating that the absorption noted with the antisera A and I was due to an immune reaction and was not a property of normal rabbit sera.

From these results it was concluded that the three antigens a, h and i of fraction C2 were all present on one peptide molecule since it had been concluded previously that each antigenic determinant was represented only once in fraction C2 as evident by immunoelectrophoresis. The significance of this conclusion will be discussed later.

8. Association of Toxicity of Crystal Digest Fraction C2 with Antigens a, h and i

In this experiment it was thought that neutralisation of the specific antigens of fraction C2 by specific antisera would indicate which, if any, of the antigenic determinants were associated with toxic function. The relationship between antigenic determinant and toxicity was investigated in fraction C2 of the berliner crystal digest by mixing separately and together the fraction with the specific antisera A, H and I. The antisera were purified and concentrated by precipitation with 50% ammonium sulphate since crude serum dried only partially on cabbage leaves and the leaf was then not acceptable to the larvae used for toxicity tests. A sufficient quantity of the specific purified antiserum was mixed with fraction C2 so that the corresponding antigen was no longer detectable by gel diffusion although it was possible to detect the other antigens. The toxicities of these mixtures were then determined at two dilutions. Controls of untreated fraction C2 were diluted to the same volume as the final volume of antiserum/fraction C2 mixtures and then 1/10 dilutions were made of both fraction C2 alone and the fraction C2/antiserum mixtures. The concentration of fraction C2 in the mixtures was thus the same in each mixture. Controls of M/100 phosphate buffer were also set up. Each sample was fed to ten larvae. The results of

toxicity tests are given below.

Table 22

Effect on toxicity by ingestion to larvae of Pieris brassicae of fraction C2 of berliner crystal digest when mixed with specific antisera

SAMPLE	UNDILUTED	1/10 DILUTION	ANTIGENS IN MIXTURE
phosphate buffer	non-toxic	non-toxic	-
normal rabbit serum	non-toxic	non-toxic	-
normal rabbit serum + fraction C2	toxic	toxic	a, h and i
antiserum A + fraction C2	toxic	toxic	h, i
antiserum H + fraction C2	non-toxic	non-toxic	a, i
antiserum I + fraction C2	toxic	toxic	a, h
antisera A and I + fraction C2	less toxic	less toxic	h

toxic = 100% mortality after 48 hr.
less toxic = 30-40% mortality after 48 hr.
non-toxic = 0% mortality after 48 hr.

The results indicate that (1) although all three antigens are apparently present on the one peptide molecule, their sites on the molecule can be selectively blocked or combined with their specific antibodies. The resulting antigen/antibody complex which is not precipitated, presumably due to antibody excess, will still react in gel diffusion with the other antisera to give

positive reactions of identity with the control antigens. Fraction C2 loses its toxicity when reacted with antiserum H so that antigen h appears to be important to the toxic activity of fraction C2 and antigens a and i alone or together have no toxic effect. When only antigen h is present, the fraction is less toxic than control fraction C2 as estimated by the number of deaths of larvae and also by the amount of food consumed. This appears to be a qualitative effect rather than a quantitative effect. The undiluted sample gave only 30-40% mortality which suggested at first that it was toxic and that its dosage had not been sufficiently great to give 100% mortality. However, the same results were obtained when the material was diluted 1/10 and fed to the larvae. Thus, although antigen h is toxic by itself, its toxicity is enhanced by a or by i so that a + h or i + h is as toxic as the control a + h + i.

The relationship between antigenic determinant and toxicity of fraction C2 will be discussed later.

DISCUSSION

The crystal-forming insect pathogen Bacillus thuringiensis has recently attracted the interest of not only bacteriologists but also entomologists and molecular biologists for several reasons. The fact that the bacterium produces a protein in crystalline form and the fact that the protein is a protoxin makes the organism and its crystal a useful tool in general studies of crystal formation of proteins and the structure of protein crystals. The protein as a protoxin is of interest to toxicologists, biochemists and enzymologists. The ease with which the organism can be cultured on simple laboratory media and the simple procedure for isolating pure preparations of the crystals are aesthetically pleasing.

The rise in interest shown in fundamental studies of the protein crystal is overshadowed by increased research into the application of the organism to controlling insect pests. Several features of the organism make it an almost ideal insecticide; it is easily cultured and mass-produced at low costs; it is pathogenic only for certain species of lepidopterous larvae and is harmless to man and animals in contrast to less-specific chemical insecticides in present use; susceptible insectsinclude many species which cause loss and

damage in agriculture, forestry and stored food products; the spores and crystals are remarkably stable; infected insects die and act as secondary sources of infection so that the organism is self-disseminating.

The following discussion presents and analyses evidence for several hypotheses and conclusions regarding the relationship between the crystal antigens and the crystal toxins. It is suggested that fundamental studies into the relationships between crystal antigenic pattern, toxicity and host-specificity are required before there can be a rational approach to the application of these crystal-formers in controlling specific insect pests.

SEPARATION OF THE SPORES AND CRYSTALS

Pure preparations of crystals were required primarily for the preparation of antisera. For antigenic analysis of crystal extracts or digests by gel diffusion it was very necessary that antibody production to antigens of the culture material other than the crystal antigens be kept to a minimum.

Completely sporulated cultures of the crystal-formers contain spores and crystals in equal numbers and a variable amount of vegetative cell material. In the selection of a suitable method for isolating crystals the following points were taken into consideration:-

(1) the final purity of crystals

(2) the final yield of crystals

(3) the time and labour involved

(4) a method for preparing spores free from crystals.

The methods described on p. 36 for the separation of crystals are based on one or both of two techniques - phase separation and flotation. The success of these techniques appears to depend on the relatively hydrophobic nature of the spore, and the relatively hydrophilic nature of the crystal. When the aqueous suspension of the spores and crystals was shaken, the spores appeared on the surface of the suspension

in the form of a scum or froth with a wax-like appearance. Also, when the suspension of spores and crystals was mixed with a hydrophobic solvent (water-insoluble) such as carbon tetrachloride or xylene, the spores were swept up by the solvent so that very few spores remained in the aqueous suspension containing the crystals.

The use of hydrophobic solvents in the isolation of the crystals of B. thuringiensis apparently arose from the use of fluorocarbon in the purification of virus particles (Bergold - 1959). In this phase system, where the purified virus remained in the aqueous phase and the contaminating protein was swept up by the fluorocarbon, it was possible, and was thought, that the system acted as a selective denaturing agent destroying the impurities and leaving the virus more or less intact (Albertsson - 1960).

The methods described in the literature (Angus - 1959); (Bateson - 1965) for the isolation of the crystals of B. thuringiensis are an extension of the methods used in virus purification. There is little evidence in these methods of any attempt to adjust the procedure used in virus purification to obtain optimum results with B. thuringiensis and to take into consideration the four points mentioned in the foregoing.

Since the two systems virus/contaminating protein and spore/crystal were rather different, it seemed logical to

suppose that the systems would require different purification methods. Accordingly, the following variables were taken into consideration as described on p.36 , although it is not suggested that these are the only variables which affect crystal purity and yield:-

(a) the solvent used in phase separation.

(b) the ratio of volume of organic solvent to aqueous suspension in phase separation.

(c) the ratio of spores to crystals in the suspension after flotation.

In (a) it was found that there was a distinct relationship between the solubility of the organic solvent in water and the purity of the crystals obtained by phase separation. From these results, it was apparent that fluorocarbon was not the best solvent, although it has a low solubility in water; xylene is less soluble in water and gave a higher crystal purity. No reference in the literature could be found which indicated this relationship between solvent solubility and the crystal purity.

While the primary consideration was given to the purity of the crystals, some attention was paid to the yield of crystals obtained at various purities. In this connection, it was found that both the nature of the solvent and the ratio of solvent to aqueous phase were important as described on p. 37 .

Removal of 60% of the spores by flotation, as described on

p. 38 , increased both purity and yield of crystals. It is quite possible that this ratio is not the optimum ratio of spores to crystals. A 50:50 suspension of spores and crystals gave both lower yields and purities of crystals and a suspension containing more than 95% crystals and less than 5% spores is very difficult to purify further by phase separation. The evidence suggests, therefore, that the most ideal ratio of spores to crystals, in the suspension to be purified by phase separation, may not be greatly different from 20:80 as used in the methods described on p. 38 .

The advantages of the methods described in this thesis over the method of Bateson - (1965) are the following:-

(1) The preliminary shaking with glass beads, as suggested by Bateson, is omitted.

(2) Only one phase separation treatment is required when a flotation technique is carried out as a preliminary (p. 38).

(3) The preliminary flotation allows preparation of pure spore preparations. The only other method, described by Angus - (1956b) for the preparation of crystal-free spores involves the use of alkali to dissolve the crystals. While the concentration of alkali used (0.02N sodium hydroxide) may not be high enough to affect viability of the spores, it

certainly causes changes in the serological properties of the spore (Doak & Lamanna - 1948).

(4) Homogenisation with xylene increases both purity and yield of crystals. Bateson (personal communication) found that, using fluorocarbon, after two phase separation treatments the yield of crystals was only 1/36th of the original. With xylene the yield of crystals may be as high as 65% of the original.

(5) The solvents carbon tetrachloride and xylene are less expensive than the fluorocarbon (trifluorotrichloroethane) used by Bateson.

(6) The use of centrifugation, as described on p.40, to separate the emulsion with xylene, gives a faster and more complete separation of the two phases and hence gives higher yields of crystals than is obtained by separation of the emulsion on standing for 15 minutes. Even after one hour standing, the organic phase of the emulsion contained interspaces of droplets of aqueous phase containing crystals.

(7) The method described under section (d) on p.40 with xylene has been applied successfully to all the isolates of B. thuringiensis except var. finitimus where the crystal is firmly attached to the spore. The method (c) on p. 40 is applicable to

var. dendrolimus. Bateson - (1965) does not mention application of his method to other strains than berliner.

These methods described on p.36 show an improvement from (a) to (d) in the purity and yield of crystals. It is not suggested that the best method used in this thesis could not be improved further if other variables such as suspension concentration and temperature were not examined. In any case, the purities of crystals of more than 99% andthe yields of up to 65% were considered adequate for the purpose of this thesis.

ANTIGENIC ANALYSIS OF SOLUBILISED CRYSTALS

The techniques of immunodiffusion have been widely applied in biology and in bacteriology in particular where bacterial antigens have attracted almost as much interest as antigenic analysis of human sera. In general, the applications of immunodiffusion involve a limited number of standard tests which include (1) analysis of the number of antigens present in a mixture (2) comparison of antisera and antigens in determinations of purity (3) determinations of antigen or antibody identity, non-identity and partial identity.

Immunodiffusion studies have secured a strong position in bacterial taxonomy since the studies of Elek - (1948,1949), Ouchterlony - (1948, 1949a,b, 1953) and Oudin - (1952) which included demonstration of pathogenicity or toxigenicity of diphtheria bacilli which could be related to the detection of the toxin in the growth medium by a double diffusion test. A strain known to be toxigenic was compared with the unknown strain in their precipitin bands with diphtheria antitoxin. If the test strain also produced the toxin a precipitin band formed which coalesced with or was continuous (gave a reaction of identity) with the precipitin band of the control strain. If the test strain was atoxigenic, the precipitin lines crossed without fusing.

Purity of anti-crystal sera

Antisera were prepared in rabbits by intravenous injection of crystal suspensions rather than crystal solutions to minimise the complexity of reaction reported by De Barjac & Lecadet - (1961) when the crystal solutions were used for immunisation. Although the crystal preparations used for immunisation were greater than 99% pure, it is quite possible that the small amount of material, including spores and vegetative cell debris, were present in sufficient amount to elicit antibody formation. Furthermore, in gel diffusion, the soluble antigen preparations certainly contained material other than crystal protein antigens since the soluble crystal preparations were prepared by suspending washed spores and crystals, i.e. whole cultures in 0.05N sodium hydroxide. Indeed, control experiments in which sporulated and partially sporulated cultures containing vegetative cells of B. cereus, which is closely related to B. thuringiensis, were suspended in 0.05N sodium hydroxide, showed that a small but significant amount of material was dissolved from the whole sporulated cultures which was precipitable at pH 4.4 and could be re-dissolved in 0.05N sodium hydroxide. However, when this material was diffused in gel against a number of anti-crystal sera, there was no reaction and so it appears that the extraneous material did not elicit sufficient antibody formation. The precipitin lines in gels with crystal solutions, therefore,

would appear to correspond to crystal antigens.

Patterns obtained by diffusion of antiserum against homologous crystal solution

As described on p. 66, many of the crystal solutions gave only one main line when diffused against their corresponding antisera, although, in several cases, there were one or more weaker lines. That is, the homologous systems are mostly monospecific and the crystals elicit in quantity a single antibody. The main precipitin lines were used to determine reactions of identity and non-identity of antigens by the criteria described in fig.8. These criteria of continuity or discontinuity of the precipitin lines could not, however, be applied to the faint lines since they were too weak and for this reason the antigenic analysis is only concerned with the main precipitin lines.

There is no conclusive evidence to indicate the significance to antigenic analysis of these fainter precipitin lines but the following possibilities present themselves:-

(1) The faint lines represent reactions between a second antibody produced in trace amounts by immunisation with the crystals and its corresponding trace antigen fully 'exposed' in the crystal solutions. This gave rise to the concept of the crystal acting as a single antigen on immunisation (Pendleton &

Morrison - 1966a). This main antigen was thought to be situated at the surface of the crystal and trace antigens responsible for the minor precipitin lines in gels were situated inside the crystal and so were not available for immunisation. The following example will expand this concept. The main antigen (surface antigen) in G 1 crystals was f and in alesti crystals was h. A solution of G 1 crystals, however, gave a reaction of identity with alesti antiserum and alesti crystal solution, indicating that antigen h was present and was revealed fully on dissolution. The hypothesis is illustrated in Table 23.

Table 23

Antigens and antibodies of galleriae G 1 crystals

IMMUNISATION			GEL DIFFUSION			
	ANTIGEN	ANTIBODY		ANTIGEN	ANTISERUM	
galleriae G 1 crystals	f++++	F++++	galleriae G 1 solution	f++++	F++++	strong
	h+	H+		h++++	H+	faint
	k+	K+		k++++	K+	faint

This hypothesis assumes that the crystal is not solubilised within the rabbit tissues. The relative insolubility of the crystal would support this. If the crystal is digested or solubilised then the concept of surface antigens must be rejected although, as will be discussed later, the rabbit-solubilised crystal may still act as a single antigen.

(2) There is one important feature of the minor precipitin lines. Apart from their lack of intensity, they are frequently more diffuse than the main precipitin lines. In this respect they bear a strong resemblance to reactions sometimes noted between some crystal solutions and heterologous antisera (Plate 4). Therefore, there is the possibility that the crystal as an antigen gives rise to only one antibody on immunisation. When the crystal is dissolved and run in gel diffusion, other antigens than the immunising antigen are exposed and may give reactions with the one antibody corresponding to the main antigen. In Table 23 on the previous page, the following could occur:-

Table 24

Antigens and antibodies of galleriae G 1 crystals

IMMUNISATION			GEL DIFFUSION			
	ANTIGEN	ANTIBODY		ANTIGEN	ANTISERUM	
galleriae G 1 crystals	f++++	F++++	galleriae G 1 crystals	f++++	F++++	strong
	h NIL	H NIL		h++++	F++++	faint
	k NIL	K NIL		k++++	F++++	faint

Thus the main line in the galleriae G 1 homologous reaction above may be represented by the reaction F++++/f++++ and the faint, diffuse precipitin line by F++++/h++++ or by F++++/k++++. Certainly, the antigens f and h are similar enough to give occasional weak reactions in gel diffusion when both are diffused against either antiserum F or H. Conversely, some homologous

systems which do not produce faint lines may be associated with antisera which are incapable of giving any reactions with antigens other than their homologous antigen. For example, when berliner crystals are used for immunisation, the antiserum A gives only one line in gel diffusion against a solution of the immunising crystals and this line in gel diffusion corresponds to the reaction A/a. A solution of berliner crystals contains antigens a, d and i. Neither antigen d nor i from other crystal solutions is capable of giving any reaction with antiserum A in gel diffusion. This may explain the absence of any minor lines due to A/d or A/i reactions. There is some evidence to support this hypothesis of the origin of minor precipitin lines although the full range of antigen/antibody reactions has not been investigated.

Although the eleven antisera have been described as monospecific, this statement needs to be qualified. As mentioned on p. 68, antiserum C contains antibody E although antigen e is not present in solutions of the crystals used to prepare antiserum C. In some way the crystals elicit antibody E during immunisation. Evidence was presented in a previous section (p.76) to show that the crystal antigens may be converted enzymatically by insect gut proteases. In a similar way the rabbit tissues may have digested the crystals, releasing antigen c which may have been converted enzymatically into

antigen e. Also the antigen e may have been present in the crystal but was lost by dissolution of the crystal in alkali. Whatever the explanation, the fact that the antiserum C contains traces of E did not affect the serotyping of test antigens since only the homologous reaction C/c was under consideration, andthe cross-reaction E/e was readily distinguishable from the main line C/c. Plate 6 shows the reactions C/c and E/e.

Attempts were made to alter the specificity of the main precipitin lines and to convert reactions of identity into reactions of non-identity. Alterations in antigen concentration, the relative distances of the wells and the pH of the crystal antigen solution had no effect on precipitin lines. Prolonged immunisation of a rabbit did not alter the specificity of the antiserum and did not increase the strength of the faint lines, where present, relative to the strength of the main line. Furthermore, some isolates were cultured on several occasions for examination of crystal solution antigens without detecting variation. Thus it was concluded that the antigenic analysis was constant and reliable and indicated real differences in the antigenic composition of the crystal proteins.

Limitations of antigenic analysis

The main antigens in some crystals may not necessarily be main antigens when they occur in other crystals, for example,

antigen c is the main antigen of isolate T 84-A but entomocidus (crystal serotype acj) has the main antigen a and antigens c and j are only exposed on dissolution of the crystal. The completeness of the antigenic analysis in detecting all the antigens in crystal solutions thus depends on the number of different "main" antigens which were detected. It is quite possible that antigens other than a-k have not been detected since only 23 of the 94 isolates were used for the preparation of antisera. This is complicated further by the possibility that some antigens may never be exposed as the main antigen for immunisation so that even if all 94 isolates were used for immunisation this may not reveal the complete complement of antigens in the crystal solutions.

The fact that immunisation with finitimus was performed with solubilised crystals and not whole crystals should not detract from the value of the results obtained with antiserum B since the main precipitin line corresponded to antigen b unique to this strain.

The suggestion that antigenic analysis may not be complete is supported further by the observation that the number of antigens detected in each crystal solution varied from one to five antigens. It would be expected that the crystal protein is sufficiently similar in all isolates for the same number of antigenic sites to occur in each isolate. The alternative is

that an apparently mono-antigenic protein such as g could more accurately be described or represented as ggg where the number of antigenic sites is three.

Immunisation with berliner crystals (serotype adi) elicits antibody A. However, when a solution of berliner crystals is used for immunisation and gel diffusion carried out with the berliner crystal solution, one precipitin line is obtained. The antiserum shows the presence of A, D and I antibodies and so is not monospecific. It may be advantageous to prepare antisera to solutions of the crystals from all the B. thuringiensis isolates and carry out absorption of these antisera with judiciously-selected crystal solutions so that monospecific antisera may be obtained. The complete range of monospecific antisera could then be used to serotype all 94 isolates.

SIGNIFICANCE OF ANTIGENIC ANALYSIS OF CRYSTAL SOLUTIONS

Significance to crystal protein structure

The gel diffusion system referred to in the previous section, i.e. antiserum to a solution of berliner crystals diffused against a solution of berliner crystals gave only one precipitin line although three different antigen-antibody reactions were represented (A/a, D/d, I/i). This suggests two possibilities; (1) the three antigens a, d and i are present on only one protein and simply represent antigenic sites on this molecule, (2) the three antigens are on a set of separate but similar molecules sharing the same basic structure and diffusion rate although differing only slightly in their respective antigens. Evidence will be discussed later which indicates that these antigens are sites on one protein molecule. Thus all the molecules from the surface to the centre of the crystal would appear to be of the same antigenic pattern and the concept of 'surface' antigens (Pendleton & Morrison - 1966c) of the crystal has less in its favour. It has already been suggested that the crystal is broken down during immunisation. If it is accepted that the crystal is broken down during immunisation, then the property of eliciting a single antibody may be more a function of the individual molecules of the crystal than a function of the surface of the whole crystal.

The fact that only one of the antigenic sites a on the

molecule is capable of stimulating antibody production suggests that the other sites (d and i) are not disclosed during immunisation with crystals. This is possibly due to the manner in which the protein molecules are coiled so that sites d and i are hidden. Dissolution of the crystals in alkali exposes these previously hidden antigens, but it is not clear how this could occur. Lecadet - (1966) concluded that alkali treatment of crystals did not lead to rupture of the peptide bonds and this conclusion is supported by Angus - (1956b) who found that the crystal protein dissolved in alkali was homogeneous. Solubilisation in alkali, however, could cause breakage of hydrogen bonds (Lecadet - 1966) and formation of a less tightly-bonded or coiled structure where antigens d and i are exposed.

One of the fundamental aspects of protein structure is the configuration of the peptide chains upon which much of the biological activity depends. Evidence as to the organisation of peptide chains in proteins has been obtained from both chemical and physical properties such as X-ray studies.

The hydration of the fibrous protein keratin may be an analogous process to the dissolution of the crystal. The untreated keratin is termed α-keratin and is in a coiled, cross-linked form; when hydrated the keratin is in an expanded form known as β-keratin (Pauling, Corey & Branson - 1951). It is less well established that in the organisation of globular

proteins the peptide chains are coiled and folded into well-defined patterns. The coiled or 'rolled-up' structure is thought to be maintained by definite cross-linkages of the types occurring in fibrous proteins. The X-ray studies of Pauling & Corey - (1951) on carbon monoxide haemoglobin have been interpreted as indicating the presence of α-helix peptide chains which may be generally present in other globular proteins.

A similar process to the hydration of keratin may occur when the crystal is dissolved in alkali so that dissolution is accompanied by an uncoiling of the protein molecule to give a change in conformation.

The importance of conformation to the serological properties of a protein was demonstrated by Berson & Yalow - (1961) who found that sperm whale insulin and pork insulin, while having identical amino acid sequences, reacted differently with some human antisera. These differences have been attributed to differences between the two insulins in the folding of the molecules and the resultant structure of surface configurations.

The crystal does not normally dissolve until the pH is raised to 12 (Angus - 1956b), but, in the presence of reducing agents which break disulphide bonds or bridges between peptide chains, solubilisation occurs at a rather lower pH of 9.5

(Lecadet - 1966). Now, when the crystal solution is precipitated at the isoelectric point 4.4, the precipitate of crystal protein can be re-dissolved readily at pH as low as 8 and alkaline crystal solutions can be adjusted readily to pH near neutrality without precipitation of the protein. This striking difference in solubility between the crystal and the precipitated protein (obtained by dissolving crystals at pH greater than 12 and precipitating the dissolved protein at pH 4.4) may be explained by a lower degree of hydrogen bonding in precipitated protein and crystal solutions and may indicate that the protein molecules in the precipitate are still in a more expanded conformation than those in the undissolved crystal. In other words, precipitation at pH 4.4 may not be accompanied by a reversal to the tightly-coiled structure of the protein molecule as it exists in the crystal.

Significance to classification and pathogenicity of B. thuringiensis strains of crystal antigens

The species or group B. thuringiensis has previously been categorised on the basis of biochemical reactions, H antigens and esterase analysis. While much of the interest of these bacteria lies in their insect pathogenicity, none of these criteria could be considered to be directly related to variations in pathogenicity within the species and to the

toxicity of the crystal protein for certain insect larvae. The antigenic analysis of the crystals may, therefore, be of value in two respects. First, previous classifications on the basis of all the criteria mentioned above have resulted in twelve sub-groups of B. thuringiensis being proposed (Norris - 1964) but classification on the basis of crystal antigen pattern or serotype alone enables thirty-six sub-groups to be distinguished among the ninety-four isolates. This may be of interest both in classification and in epidemiological studies where isolates from similar sources have to be compared. Second, there is considerable interest at present in the use of sporulated cultures of B. thuringiensis as a control agent for certain insects by their application in the field. Since the toxin crystal appears to be an essential component of the insecticide and there appears to be a degree of host-specificity in the bacterium, the crystal serotype may be important in determining beforehand the suitability of an insecticide preparation based on a culture of B. thuringiensis for a certain species of insect pest. Such an approach would be more rational than the trial-and-error system presently applied to the selection of suitable pathogens. Variations in the pathogenicity of various isolates may reflect differences in the molecular structure of the toxic crystals which in turn may correspond to antigenic differences in the crystal protein. As will be discussed later, differences

in the antigenic patterns of the toxin of the protoxin crystals can also be detected between different strains. An example will illustrate the possible relationship between crystal antigens and pathogenicity. The isolates entomocidus and subtoxicus, numbered 40 and 41 in the collection of Norris - (1964), were identical on the basis of their biochemical reactions, flagellar antigens and esterase patterns. They differ greatly in pathogenicity for the silkworm Bombyx mori (Heimpel & Angus - 1958); entomocidus was about 100 times more toxic than subtoxicus. Burgerjon - (1960), however, found that almost the reverse was true when Pieris brassicae was the assay insect. The difference in toxicity may well correspond to antigenic differences between the crystals. Entomocidus has the crystal serotype acj and digest pattern ahij; subtoxicus has the crystal serotype a and digest pattern ah.

On the whole, however, it is very difficult to draw any conclusions about any relationship between antigenicity and toxicity simply because the necessary corroborative work in comparative toxicity has not yet been carried out. Such work is obviously very essential since there are no toxicity results which would indicate that different isolates of B. thuringiensis with identical serotypes have similar toxicities or that antigenic composition is necessarily indicative of toxicity.

ANTIGENIC ANALYSIS OF FRACTIONS C1 AND C2 OF CRYSTAL DIGEST

Fractionation of digest

When crystals were digested by the gut juice of *Pieris brassicae* and the digest fractionated on 'Sephadex', two components were separated - fraction C1 with molecular weight greater than 200,000 and fraction C2 with molecular weight 5,000-10,000. Fraction C2 was toxic on both injection and ingestion but fraction C1 was toxic on ingestion only (p.92).

The technique of enzymatically digesting proteins followed by examining the digests for serologically active fragments has been employed widely. For example, Kaminski - (1962) investigated antigenic relationships between hen and duck ovalbumins, before and after proteolytic digestion.

Antigenic analysis of the five fractions obtained by column chromatography of digests of protein extracted by alkali from the crystals was not carried out. As will be discussed in the following section, this situation was hardly comparable to the digestion of whole crystals and accordingly was not thought to have any relevance to studies of the digestion of the crystals *in vivo*.

As described on p. 76 , antigenic analysis with the eleven antisera available (A-K) of fraction C1 of the digests of a number of different crystal serotypes indicated that the antigens

present in solubilised crystals were the same as those present in fraction C1 of the crystal digest of each isolate. This evidence suggests that fraction C1 corresponds closely to solubilised crystal protein and this is supported by molecular weight estimations on 'Sephadex' G-200 where both are exluded (molecular weight greater than 200,000) and by the lack of toxicity of both fraction C1 and alkali-solubilised crystal protein when injected into larvae. However, the digestion products of fraction C1 are not the same as those of the alkali-solubilised crystal protein so there may be subtle differences in conformation. This will be discussed later.

Antigenic analysis of fraction C2

The antigenic analysis of fraction C2 of the crystal digests of 93 isolates of B. thuringiensis by the same method for the antigenic analysis of solubilised crystal protein indicated a complexity of antigenic patterns (p. 77). The method of preparation of fraction C2 of these isolates, i.e. by a secondary digestion of the crystal digest, is described on p. 45.

The idea behind an antigenic analysis of fraction C2 was that the antigenic patterns of this fraction, which is believed to represent or contain the activated toxin produced by digestion, would bear a closer relationship to variations of toxicity of the crystals from different isolates than would analysis of alkali-

solubilised crystals or analysis of fraction C1. There is as yet, however, no evidence to support such a relationship between the toxicity of an isolate and its fraction C2 antigenic pattern.

The results of fraction C2 analysis indicate only a very general relationship between antigenic pattern of fraction C2 and solubilised crystals. It would not be possible to predict with any certainty the antigens which would be present in fraction C2 of an isolate of known crystal serotype since different crystal serotypes often produce the same antigens in fraction C2 of their digests. For example, isolates AC 58 (crystal serotype dei) and galleria allemand (crystal serotype i) have fraction C2 patterns i. Again, the one serotype occurring in several strains may produce a variety of antigens in fraction C2, for example, isolate ACC 1731 and Ashman have the crystal serotype e but their fraction C2 patterns are ci and i respectively.

Relationship between antigens of fraction C2 and toxicity

The relationship between toxicity and antigenic pattern of fraction C2 may require to include consideration of the source of the enzymes employed in digesting crystals. That is, the results of fraction C2 analysis of the 93 strains may only be relevant to the toxicity of these strains to Pieris brassicae.

Insects which are not susceptible to the crystals could, theoretically, be grouped as follows:-

(1) insects which do not digest the crystals.

(2) insects which digest the crystals without formation or release of the toxin; also insects which produce different antigens in fraction C2 than does Pieris brassicae.

(3) insects which release toxins and antigens but, nevertheless, are not susceptible.

Thus toxicity of a crystal type for an insect may depend on the interaction of three factors:- crystal serotype, gut enzymes and the presence of the susceptible tissues in the insect.

Possible importance of protein conformation to antigenic pattern

The reason for one crystal serotype producing a variety of antigens on digestion may be explained by differences in the conformation of the protein molecules in the crystal which may be sufficient to influence the enzymatic digestion without causing detectable antigenic differences in the crystals when solubilised by alkali. Thus in the berliner group, the crystal serotype adi generally produces ahi in fraction C2 and in a few cases ai or ah. It is postulated that the crystal serotype adi can exist in three conformations in the crystal, the most usual conformation giving ahi and two others which, owing to the manner in which the peptide

chains are linked, are digested in such a way that loss of an antigen occurs.

There is some evidence, however, that differences in conformation of possibly the one protein may affect antigenic pattern of the solubilised crystals. For example, isolates having the crystal serotype a give fraction C2 patterns ai (1 isolate), ah (3 isolates) and ahi (1 isolate) which are all typical of fraction C2 patterns of crystal serotype adi. In the case of crystal serotype a, it seems possible that antigenic sites other than a exist on the protein molecules but are unexposed even by solubilisation of the crystals by alkali, so that the crystal serotype a retains its tightly-coiled conformation to a great extent during alkali solubilisation of the crystal. The other possibility is that solubilisation in alkali causes changes in conformation sufficient to affect the antigenicity of the antigenic sites. The relationship between crystal serotype proteins a and adi is supported by the fact that antigen d could sometimes be detected in gel diffusion in the isolate Bakthane (crystal serotype a) when the crystal solution was strong.

The isolate E 1 of the berliner group is unusual in possessing the crystal serotype e. Crystals of this isolate, when injected into a rabbit give an antiserum corresponding in type to E. A solution of the crystals diffused against the

antiserum E gives a precipitin line which is rather less strong than is obtained with crystal solutions from isolates of the kenya group which more typically contains e than the berliner group. Curiously, digestion of E 1 crystals gives fraction C2 ahi which is more typical of digestion of adi, e.g. isolate berliner. Thus the undigested protein of E 1 may not be as different to adi as its crystal solution antigen suggests, and the presence of antigens a, d and i in E 1 in an unexposed form is strongly suggested. It is proposed that none of the antigens a, d and i are exposed during immunisation or during alkali solubilisation through some feature of the conformation of the protein. The antigen e in these circumstances may represent an antigen partly-exposed during immunisation such as a.

It is not suggested, however, that all those crystal serotypes which produce the same antigenic pattern in fraction C2 are necessarily the same protein molecule in different 3-dimensional arrangements. Differences may be due to different amino acid sequences in the crystal protein. It is apparent that antigenic sites in the crystal may be destroyed by enzymic action during digestion; the antigens b, d, f and k have not been detected in fraction C2. Antigen d is frequently replaced by h suggesting that the part of the peptide chain containing antigenic site d contains a peptide linkage hydrolysable by the digestive enzymes of Pieris brassicae.

Digestion of site d then leaves a residue corresponding to antigen h. Landsteiner - (1945) found that changes in specificity of an antigenic site on a protein could be related to loss or gain of residues similar in size to amino acids. The alteration of antigen d to h may represent a loss of only a terminal amino acid residue. However, in many cases, antigens in crystal solutions disappear on digestion and it is possible that the residual material, possibly representing only a slight modification of the original peptide, may not correspond to any of the eleven antigens in the crystal solutions. So that if immunisation with fraction C2 was carried out more than the seven antigens could be detected in all ninety-three strains.

The relatively high frequency with which antigen i appears in fraction C2 deserves mention. The fact that this antigen commonly appears in place of other antigens present in crystal solutions suggests that it represents a structure basic to several antigens, i.e. it may be the 'backbone' or 'core' of several antigenic determinants.

One further point is of interest and will be discussed later. The fact that any antigens can be detected at all in fraction C2 is surprising when the relative ratio of molecular size of fraction C2 to fraction C1 is considered. Digestion causes a decrease in molecular weight from more than 200,000

to less than 10,000, i.e. a reduction in molecular weight of between 1/20 and 1/40. On the basis of random enzymatic breakdown, the chances of one antigen surviving is very small. The possibility that the antigenic sites in fraction C1 are repeated along the length of the peptide chain will be discussed later.

DIGESTION OF CRYSTALS AND SOLUBILISED EXTRACTS

As reported on p. 92 , different results were obtained when crystals were digested and when solubilised crystal preparations were digested. These differences in results will be discussed in this section.

Digestion Of Crystals

Digestion of crystals resulted in only two fractions which were both toxic on injection, although only the low molecular weight fraction (fraction C2) was toxic by injection. It has been suggested (Angus - 1956b) that the crystal is a protoxin and Lecadet & Martouret - (1962) found that digestion of crystals caused appearance of a soluble fraction toxic by injection. Fraction C1 was shown to be an intermediate digestion-product between crystal and fraction C2 and no toxic material of molecular weight lower than 5,000 molecular weight was detected. Thus it is concluded that fraction C2 contains the toxin or toxins and that fraction C1 is an intermediate which is not fully activated and so is not toxic by injection.

It is of interest to compare these results with those of Lecadet & Martouret - (1962) described on p. 7 who also distinguished two fractions in crystal digests which they referred to as the 'dialysable fraction' and the 'non-dialysable' fraction. It seems very likely that the two fractions of

Lecadet & Martouret - (1962) correspond to the two fractions reported in this thesis. Certainly, on the basisof molecular weight estimations, the non-dialysable fraction (greater than 45,000 molecular weight) could correspond to fraction C1 (greater than 200,000 molecular weight) and the dialysable fraction (less than 12,000-10,000 molecular weight) to fraction C2. Now, although both fractions of Lecadet & Martouret - (1962) were toxic on ingestion, which agrees with the results on p. 92, these results show that fraction C1 is scarcely toxic on injection, whereas both fractions as reported by Lecadet & Martouret - (1962) were toxic on injection. From the table on p. 8 , it can be seen that Lecadet & Martouret - (1962) found the non-dialysable fraction rather less toxic on injection than the dialysable fraction. While the two fractions had similar toxicities on ingestion, the dialysable fraction killed 100% of the larvae on injection at a concentration as low as 22.4 µg. N/ml., but the non-dialysable fraction required to be at a concentration of 225 µg. N/ml. to achieve the same kill. Thus the difference in toxicity on injection is a factor of at least 10 times.

It is quite possible that the non-dialysable fraction of the digest contained traces of the dialysable fraction so that the non-dialysable fraction was apparently toxic on injection. Lecadet & Martouret - (1962) make no reference to the procedures

involved in their dialysis fractionation or to any checks that dialysis had been continued long enough to completely free the non-dialysable material of the dialysable fraction. Also it is conceivable that digestion of the non-dialysable fraction continued during and after dialysis. The digestive enzymes responsible for crystal digestion are thought to have molecular weights of 32,000 (Lecadet & Dedonder - 1964a) which is too large to allow their removal from the non-dialysable fraction. The work described on p.90 showed that fraction C1 is readily converted into fraction C2 even at $4^{o}C$ which is a temperature commonly used in dialysis procedures, so that it may be impossible to prepare pure non-dialysable fraction by dialysis. Isolation of fraction C1 would be more satisfactory by exclusion on 'Sephadex' G-200 since the digestive enzymes would be in the included fraction on this medium and so would be prevented from acting further on fraction C1 by virtue of their separation from fraction C1. The results on p. 91 suggest that fraction C1 is very slightly toxic on injection and again these results could derive from traces of fraction C2 being present as a result of imperfect column chromatography, although the possibility cannot be ruled out of spontaneous breakdown of labile peptide linkages in the fraction C1 to produce fraction C2.

Digestion of solubilised crystals

The results are described on p. 83 and show that there are two important differences when compared with the digest of crystals - (1) a wider range of digestion products is obtained, (2) the results of toxicity tests on the individual fractions are less conclusive in some fractions.

In contrast to crystal digest fractions, the highest molecular weight fraction E4 is moderately toxic on injection, while some of the low molecular weight fractions (E3, E5 and E6) are not toxic on injection. Fraction E2 corresponds approximately in its molecular weight to fraction C2 of the crystal digest but is only moderately toxic andthen only by injection. Apparently, only fractions containing protein over 200,000 molecular weight (fractions E1 and E4) are toxic by feeding although this material is also moderately toxic by injection. An interesting finding is that a mixture of the four lowest molecular weight fractions (E2 + E3 + E5 + E6) was at least as toxic as the whole digest.

The sequence of formation of digestion products was not determined and so it is not possible to state which, if any, fractions are intermediate digestion products. The results, nevertheless, show that the high molecular weight material (E4 and E1), which very probably contains some intermediate digestion products, is partially activated since it is toxic

on injection. The smaller fragments (E3, E5, E6) which possibly derive from these intermediates are not toxic. That is, only the large molecules (with the possible exception of E2) appear to be truly toxic on injection.

From these results, it is possible that digestion proceeds in such a manner that the toxic groups are hydrolysed at an early stage in the digestion which results in several differently-sized peptides each bearing an incomplete toxic group. In the case of fraction E2, this may be sufficiently representative of the original toxic grouping to cause some toxic effect on injection. The lack of toxicity of this fraction on ingestion suggests that it may be rapidly digested to non-toxic fragments such as fraction E3. The toxicity of the mixture E2 + E3 + E5 +E6 suggests that the components, individually not toxic (with the exception of E2), are capable of acting together so that the mixture of the fragmented toxic groups are together representative of the toxic group. It seems unlikely that the separate parts of the toxic group recombine chemically but one could imagine that the toxic group consists of several sites on the molecule which require to act together to give a toxic effect. It may not be important whether these sites are on one peptide or on separate peptides; they may only require to be present in a mixture to be toxic.

There is some evidence that the toxicity of the alkali solubilised crystal protein is lower, weight for weight, than the

untreated crystals. Angus - (1956b) found that more potent extracts were obtained with mild alkali than with strong alkali solution; and the toxin was more easily inactivated by heat when in solution than it is in the crystal. Dissolution of the crystals in alkali caused a loss of 93% of the toxicity of protein. Thus alkaline extraction may be responsible for the low toxicity of some fractions of crystal extract digest.

General considerations of digestion

On the whole, the picture that emerges is that alkali-extracted crystal protein is digested in a more haphazard manner than are the crystals. It is only possible to speculate on reasons for this difference but this effect may be related to the ideas on protein conformation which developed from antigenic studies. It was concluded that alkali alters the conformation of the crystal protein by expanding the tightly-coiled protein in the crystal. Thus most of the peptide bonds hydrolysable by the insect gut enzymes are unexposed in the tightly-coiled protein but are exposed in the expanded protein. Also out of steric considerations the tightly-coiled form would have fewer peptide linkages freely accessible to enzyme molecules. Presumably the toxic groups are preserved intact by digestion of the tightly-coiled form.

Lecadet & Dedonder - (1964b) reported the presence of two proteases in the gut juice of Pieris brassicae which were

differentiated by their substrate specificities. One of the enzymes hydrolysed peptide linkages at the carboxyl of arginine (trypsin-like) and the second was active at aromatic linkages (chymotrypsin-like). These two enzymes may be the principle enzymes involved in activation of the crystal protoxin. It is not known whether only one is required for activation or whether they act together. In the tightly-coiled form of the protoxin there may be linkages which are accessible to only one of them and in the expanded form there may be a greater number of linkages accessible to both enzymes. Since the specificities of the two enzymes are different, the expanded form would yield a greater variety of digestion products.

One feature of digestion of berliner crystals with Philosamia cynthia gut juice is interesting in this connection. Digestion of crystals gives fractions C1 and C2 with antigenic patterns identical to those obtained with the gut juice of Pieris brassicae. Pieris brassicae and Philosamia cynthia are not related insects; the former is a butterfly and the latter is a moth, and they belong to separate super-families in the Order Lepidoptera. It is unlikely that their gut proteases are identical or even have identical specificities. Furthermore, the number of susceptible insects is now greater than 100 species. The structure of the protoxin molecule may, therefore,

be as important as the proteases in determining the digestion products if different proteases give identical products. It is surprising that digestion of the crystal does not give a greater variety of digestion products in comparison with other proteins. Ingram - (1958) digested purified haemoglobin with crystalline trypsin and obtained twenty-six different peptides.

IMMUNOELECTROPHORESIS AND GEL DIFFUSION OF DIGESTS OF BERLINER CRYSTALS

The antigens a and i are present in both fraction C1 and fraction C2 of the berliner strain. Gel diffusion of the unfractionated digest (fraction C1 + fraction C2) against antisera A and I showed that the control antigens a and i branched into two lines each giving a reaction of identity with the control antigen of solubilised crystals (Plate 8). A similar phenomenon has been reported by Pope & Stevens - (1958) for chemically hydrolysed diphtheria toxin where the hydrolysed material contains several fragments bearing the original antigenic site. Since fraction C1 and fraction C2 have widely different molecular weights, their diffusion rates are correspondingly different and two precipitin lines are formed by diffusion against each antiserum A or I. These results were not taken as indicating that there were only two molecules containing a or i in the digest since two or more molecules of similar molecular weights may diffuse at similar rates to give apparently only one precipitin line.

On this basis, immunoelectrophoresis was applied to fraction C1, fraction C2 and the unfractionated digest. As described on p. 95, this technique was not able to resolve the digest into more than two precipitin arcs each for antigens a and i and one arc for each of the antigens d and h. The following conclusions were drawn:-

(1) the whole digest can be represented antigenically by

fraction C1 + fraction C2.

(2) fraction C1 contained probably only one molecule represented antigenically by adi.

(3) fraction C2 contained probably only one molecule represented antigenically by ahi.

Each antigen was present singularly in each fraction as determined by the single arc for each antigen in each fraction. While these results strongly suggested that each fraction consisted of one molecule carrying three antigenic determinants, the results at this stage were not regarded as conclusive. In combination with the results of electrophoresis in polyacrylamide gel (p. 96) they are certainly good evidence for such a concept.

The fact that fraction C2 appeared to be the ultimate toxic unit, since no toxic fractions of lower molecular weight were detected, made it more interesting than fraction C1 which was simply its precursor in digestion. For this reason, determination of whether the antigens were on the same or separate molecules in fraction C2 was regarded as more important than such a determination of fraction C1. For reasons to be explained later, the results with fraction C2 enable a logical deduction to be made about the number of antigenic molecules in fraction C1.

REACTION OF FRACTION C2 WITH ANTISERA A AND I

This technique is described on p. 51. The success of the method depends on the differences in molecular weight between fraction C2 antigens and antibodies A and I. Fraction C2 was reacted with the antisera individually and with normal rabbit serum. Material which had not combined with the antibodies or with normal serum was included in 'Sephadex' G-50 and tested for antigens a, h and i. The author is not aware of this technique having been applied previously in the field of molecular biology and immunology.

The interpretation of the results is that each monospecific antiserum is capable of combining with all antigenic material in fraction C2. All three antigens were withdrawn from fraction C2 by one antiserum indicating that the three antigens were on the one molecule.

The remaining 15% or less of fraction C2 which was non-antigenic could have derived from any or all of the following:-

(1) side-reaction products from the breakdown of fraction C1 to fraction C2.

(2) protein, peptide or aromatic amino acids or any other materials absorbing at 280 mμ present in the crude gut juice of Pieris brassicae.

(3) products of digestion of fraction C2.

There are some results which may appear to be in conflict

with these observations and conclusions. When each of the antisera A, H and I are mixed individually or in pairs with fraction C2, it is found that the other antigens are still detectable by gel diffusion tests on the mixture. From this it would appear that the antigens are not linked but the complex ahi/A, for example, could, if not precipitated, be able to diffuse in gel diffusion to give a reaction with antisera H and I since the antigenic sites h and i would not be blocked. From steric considerations, the large antibody molecule (greater than 50,000 molecular weight) could interfere with adjacent antigenic groups on such a small molecule as 5,000-10,000 molecular weight. However, the number of antibody molecules which may combine with one molecule of antigen is relatively large, for example, Najjar & Fisher - (1956) found that sixteen molecules of antibody combined with one molecule of ovalbumin of molecular weight 44,000 and ten molecules of antibody combined with one molecule of alcohol dehydrogenase of molecular weight 96,000 (Harshman, Robinson & Najjar - 1963).

The conclusion that antigens a, h and i in fraction C2 of the berliner digest are present on one peptide only, strongly implies that the antigens a, d and i are present on one molecule in the crystals.

RELATIONSHIP BETWEEN TOXICITY AND ANTIGENS IN FRACTION C2

The experiment with berliner fraction C2 is a preliminary to a comparative study of antigenically distinct crystals in an attempt to relate toxicity to particular antigens or combinations of antigens. The results on p.100 indicate that in Pieris brassicae neutralisation of some antigenic determinants reduces toxicity partially or completely. It is not suggested that each antigen represents a toxic group or a particular function in toxicity but it is suggested that the toxic group may be associated in particular with antigen h. The toxic group may not be precisely the same as the antigenic site h and may involve a greater or lesser part of the molecule than the antigenic site h. Similarly, although the active centre of an enzyme molecule is generally considered to be small, about the size of the substrate, the whole enzyme molecule appears necessary for function since removal of small parts of the molecule distant from the active centre may adversely affect function.

It is thought that antigenic site h is closest of the three antigens to the toxic group. Neutralisation of sites a and i may affect the activity of the toxic group in a similar manner to reduction in enzyme activity by removal of small portions of the enzyme molecule.

Although h is toxic alone, there appears to be an accentuation of the toxicity when either or both a and i are not blocked by their specific antisera. Also, antigens a and i together or separately arenot toxic.

THE CRYSTAL PROTOXIN AS A POLYMER OF THE TOXIN

There are several pieces of evidence which give strong support to the concept that the protoxin is activated by a depolymerisation process. The points are as follows:-

(1) The results on p. 97 indicate that the crystal is digested to give a single molecule of toxin representing a possible 85% of the digest. In this sense, the digestion is extraordinarily specific. In other words, if the protoxin was not a polymer of the toxin only $\frac{5,000\text{-}10,000}{200,000} \times 100\% = 2.5 - 5\%$ of fraction C2 would be in the form of antigenic material or toxin.

(2) There was no material in the digest intermediate between fraction C1 and fraction C2 and very little material of molecular weight less than fraction C2.

(3) Many antigens survive digestion. As suggested earlier, the considerable difference in molecular weight between fraction C1 and fraction C2 would militate against antigen survival. It is suggested that the antigens of fraction C1 are repeated along the length of the molecule in the form aidaidaidaid.... Depolymerisation between d and i linkages may alter d and convert it to h.

(4) Lecadet & Martouret - (1962) in a comparison of the dialysable fraction (probably fraction C2) with the non-dialysable fraction (fraction C1) found that weight for weight they were almost equally toxic on ingestion. The slightly greater toxicity of the

dialysable fraction (activated toxin) may be due to its ability to act immediately without requiring activation in the gut.

While it is known that certain proteins exist as polymers of a simpler protein or peptide, this phenomenon is poorly documented and is generally associated with the hydrolysis of collagen to the unit gelatin.

A possible analogy to the activation of the crystal protoxin of B. thuringiensis is the toxin of Clostridium botulinum referred to as botulinum toxin. The type A toxin has a molecular weight of 900,000 (Lamanna & Doak - 1948) and type B has a molecular weight of 60,000 (Lamanna & Glassman - 1947). Both types A and B are similarly toxic weight for weight. The fact that these toxins frequently act by being absorbed through the alimentary tract is interesting since generally only small peptides are capable of being absorbed by this route. It has been suggested that the toxin is in the form of a protoxin which is enzymatically digested to release smaller toxic fragments. Trypsin has been shown to cause a great increase in toxicity of cultures of type E Clostridium botulinum (Sakaguchi & Sakaguchi - 1959). Furthermore, type A toxin dissociates partially at pH 7.5 to give a toxic fragment of molecular weight 71,000.

CONCLUDING REMARKS

The work described in the preceding pages has thrown an interesting light on the complexities of the crystal protein of the B. thuringiensis group of bacteria. Prior to the present work, there had been little investigation of the possibility that the nature of the crystal might be different in different strains. This thesis has shown that differences do exist.

As in all research, more problems have revealed themselves than have been solved. The author hopes that in the future, he may be able to investigate such topics as the relationship of crystal and crystal-digest antigens to host-specificity and to the manner in which the toxin acts on insect tissues.

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* Pendleton, I.R. & Morrison, R.B. (1966a) - J. appl. Bact 29 519-528.

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