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The Amino Acid Composition of some Insect Viruses and their Characteristic Inclusion-body Proteins*

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The majority of known insect virus diseases are characterized by the production of immense numbers of inclusion bodies. These are of two main types: polyhedra, each of which contains large numbers of virus particles, and oval 'capsules', each of which contains a single virus particle. These inclusions vary in size and shape with the virus and host insect (Bergold, 1953). After dissolving them in very weak alkali, about 1.5-5% of the weight is recoverable as infectious virus. The remainder consists principally of protein which is homogeneous in the ultracentrifuge, and is of molecular weight varying from 275 000 to 375 000, depending on the host (Bergold, 1947).

There have been few previous analyses of the amino acids of insect viruses or their inclusion-body proteins. Desnuelle, Chang & Fromageot (1943), and Desnuelle & Chang (1943, 1945) reported a partial quantitative analysis of what was thought to be the virus of *Bombyx mori* L. (silkworm), but which later proved to be the non-infectious polyhedral protein of this virus (Bergold, 1953). More recently, Yoshitake, Aruga & Suzuki (1950) made a qualitative analysis of the *Bombyx* polyhedra, and Wellington (1951) made a qualitative comparison of the amino acids of both *Bombyx* and *Cacoecia murinana* Hb. viruses and their respective inclusion-body proteins. In addition, a comparative quantitative study of the proteins of the silkworm virus, its membranes, and its polyhedra has just been concluded (Bergold & Wellington, 1954).

The quantitative paper chromatographic method (Wellington, 1952, 1953) used in this last study is sufficiently sensitive and accurate to permit analysis of minute amounts of virus material for comparison of the results with those of other workers who have

analysed the viruses for other constituents. For example, Wyatt (1952) studied the deoxypentose nucleic acids (DNA) of a number of insect viruses and, although he found no general parallelism between DNA composition and biological relationship, he did find a discontinuous distribution of molar ratios of adenine plus thymine: guanine plus cytosine among the viruses. It seemed worth while to determine whether variations in amino acid composition would bear any relationship to this distribution. It was also of interest to know whether insect viruses would exhibit variations in amino acid composition similar to those reported by Knight (1947) for different strains of tobacco mosaic virus. Qualitative work such as that reported earlier (Wellington, 1951) has never shown any such differences.

In addition to clarifying such relationships, it was hoped that quantitative analyses might also show both the relationship of the virus to its specific inclusion protein, and the interrelation of the various inclusion-body proteins.

MATERIALS AND METHODS

Preparation of the viruses. Viruses were isolated from purified inclusion bodies by dissolving these in very weak Na_2CO_3 followed by centrifuging, as described by Bergold (1947, 1953).

No attempt was made to isolate the protein fractions of the viruses for two reasons, firstly because the amount of material available was so small, and secondly because Wyatt (personal communication) had not succeeded in separating the nucleic acid fraction of *Porthetria dispar* (L.) virus from the protein except by partial hydrolysis of the protein. Therefore it was assumed that the protein-nucleic acid linkage was of a similar firm type for all the insect viruses. In addition the virus preparations all contained virus membranes of the type recently described for the virus of *Bombyx mori* (L.) by Bergold & Wellington (1954).

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60 to 100 mg. of inclusion bodies yielding 1.5–3.0 mg. virus were used for each analysis. For hydrolysis, each virus preparation was sedimented and dried (3 days *in vacuo* over P_2O_5) in a 6 × 50 mm. glass culture tube.

Preparation of inclusion-body proteins. The inclusion-body proteins were recovered from the alkaline solutions by precipitation at the isoelectric point (approximately pH 5.5–5.7) with dilute acetic acid, followed by repeated washing with distilled water, or by dialysis against distilled water for one week at 4°. For hydrolysis, approximately 3 mg. of each preparation were dried for 3 days *in vacuo* over P_2O_5 .

Hydrolysis. Each sample was hydrolysed with 50 μ l. constant-boiling HCl (twice distilled from glass) per mg. in a sealed glass tube for 16 hr. at 105–108°.

Oxidation. The acid hydrolysate was split into two portions. To one was added 0.1 vol. 30% (w/v) H_2O_2 and the mixture was thoroughly stirred and left at room temp. for 2 hr.

Chromatography. Two-dimensional chromatograms were run by the ascending method on sheets of Whatman no. 1 paper measuring 30 × 28 cm., to which 10 μ l. portions of the acid hydrolysates, corresponding to 180–200 μ g. of the original sample, were directly applied. Analyses were carried through in triplicate and the results were averaged. Chromatograms of oxidized and unoxidized hydrolysates were run with different solvents, since, at the time this study was undertaken, no pair of solvents was known which satisfactorily separated all the amino acids on a single small two-dimensional chromatogram.

The chromatograms of the oxidized samples were carried through the entire procedure exactly as previously described (Wellington, 1952). A mixture of abs. ethanol (60 vol), *tert.*-butanol (20 vol.), reagent NH_3 of sp.gr. 0.90 (5 vol.) and water (15 vol.) was used for the first run. Draper & Pollard's (1949) modification of the phenol solvent of Consden, Gordon & Martin (1944) was used for the second run in an atmosphere containing cyanide and NH_3 . This procedure separated all the amino acids except leucine, isoleucine and phenylalanine. Cysteine and/or cystine were determined from these papers as cysteic acid, and methionine as methionine sulphone. The degree of oxidation of tyrosine was not easily controlled, so that accurate determination of this amino acid from the oxidized hydrolysate was difficult.

In order to overcome this difficulty, and also to allow estimation of phenylalanine separately from leucine and isoleucine (which it overlaps with the solvent pairs used for the oxidized samples), chromatograms of unoxidized samples were run. For these, the solvents described by Redfield (1953) were used, modified by substitution of NH_3 for diethylamine, since an autoclave with an outside vent, suitable for removal of diethylamine, was not available. This gave excellent separation of tyrosine, phenylalanine and leucine plus isoleucine. If the quantities of amino acids on the paper were not too large, separation of leucine from isoleucine was also achieved. These chromatograms provided an additional check on all the other amino acids except lysine and arginine, which are not separated by the modified solvent.

The chromatograms were sprayed with 2% ninhydrin in absolute ethanol. Colour development proceeded under controlled conditions ($20 \pm 1^\circ$ and $38 \pm 2\%$ relative humidity; Wellington, 1952). Control of humidity is especially important since it has been shown (Wellington, 1953) that there

is only a very narrow range of relative humidity (35–40%) at 20° within which the maximum colour reaction of most amino acids with ninhydrin on paper can be expected, and that the colour intensity falls off rapidly on both sides of this maximum. Furthermore, it is important to hang the papers in a good draught after the final chromatographic run and before spraying with ninhydrin, so that the NH_3 is completely removed: otherwise, variable results are obtained. 24 hr. were allowed for this step. After colour development, elution and estimation of the amino acids were carried out as previously described (Wellington, 1953).

A number of the amino acids gave a slightly higher colour yield after chromatography with the modified Redfield solvent than they did when phenol was the second solvent. Consequently, although the same standard curves could be used for valine, glutamic acid and serine separated by either method, new standard curves had to be prepared for the other amino acids. Once prepared, these were reproducible from day to day.

Proline analyses. Proline analyses by the method used for the other amino acids are not very reliable. The method of Chinard (1952) applied to proline from chromatograms run with phenol: $CN^-:NH_3$ as the second solvent, and sprayed with ninhydrin, gives reproducible values only when based on a simultaneously run standard. This was done for all but the last few analyses. In these, it was found that results, reproducible from day to day, could be obtained after spraying with ninhydrin, by using the Chinard reaction on proline spots cut from chromatograms run in the modified Redfield solvent.

Tryptophan analyses. Tryptophan was identified by the method of Gordon & Mitchell (1949). This technique is satisfactory for pure proteins, but, like all the other tests for tryptophan that were tried, it is of no use in the presence of deoxyribonucleic acid. Consequently the presence or absence of tryptophan was not determined in the virus samples.

RESULTS

The results of the analyses of a number of inclusion proteins are given in Table 1, as g. amino acid recovered/100 g. sample weight. The total recoveries calculated as residues would more nearly approach 100% if tryptophan analyses had been quantitative. Further, there was considerable residue in each hydrolysate. Whether this was from unhydrolysable material or humin formed during hydrolysis is not clear since the inclusion-body proteins were not soluble in the constant-boiling HCl used for hydrolysis.

The results for the analyses of the virus samples are given in Table 2. No attempt was made to compare the amino acids recovered on a basis of sample weight. It was thought more meaningful to express the values for each amino acid as a percentage of the total amino acids recovered, especially since the amino acids recovered from the *Porthetria dispar* (L.) (*Pd*) virus accounted for so much less of the sample weight than those of the other viruses. Further, since the analyses were of the total virus and, except for the virus of *Bombyx mori* L. (*Bm*), no data are available on the percentage of virus

Table 1. *Amino acid composition of inclusion-body proteins*

Estimated by paper chromatography of acid hydrolysates, and expressed as g. amino acid/100 g. material analysed. Tryptophan, also present in these proteins, was not estimated.

No. of hydrolysates analysed ...	Polyhedral type*							Capsule type* <i>Cm</i>
	<i>Pd</i>	<i>Cf</i>	<i>Ma</i>	<i>Md</i>	<i>Bm</i>	<i>Cpe</i>	<i>Ns</i>	
	3	3	2	2	5	2	2	
Amino acid								
Aspartic acid	11.1±0.35†	12.2±0.27	13.5	13.6	12.5±0.29	13.3	12.4	11.7±0.30
Glutamic acid	13.2±0.30	12.2±0.33	12.4	11.7	12.7±0.30	12.8	13.5	12.9±0.33
Histidine	3.7±0.44	3.2±0.23	2.8	2.2	2.8±0.18	2.8	3.6	1.7±0.17
Lysine	8.5±0.29	9.8±0.41	9.4	6.3	10.5±0.25	8.7	6.6	6.3±0.21
Arginine	9.2±0.26	7.9±0.29	9.7	9.4	6.8±0.33	10.1	10.3	10.1±0.41
Glycine	3.1±0.12	3.0±0.15	2.7	2.8	3.3±0.09	3.0	3.6	2.8±0.17
Alanine	3.3±0.26	2.8±0.18	2.9	2.8	2.9±0.05	2.8	2.6	2.8±0.12
Valine	6.0±0.24	7.2±0.04	6.9	5.8	5.7±0.16	7.0	5.7	5.2±0.21
Leucine and/or isoleucine	13.0±0.46	12.3±0.38	14.3	13.4	13.3±0.20	13.2	14.7	14.9±0.20
Proline	4.9	5.7	8.2	6.0	6.0	8.3	5.2	5.4
Phenylalanine	7.5±0.66	7.9±0.20	7.0	7.1	6.9±0.20	6.4	9.5	9.4±0.52
Tyrosine	10.0±0.23	9.4±0.27	9.6	10.1	10.9±0.21	7.6	5.3	7.2±0.38
Serine	3.8±0.07	3.4±0.07	3.6	4.3	3.3±0.07	3.6	3.6	3.4±0.15
Threonine	4.7±0.15	3.3±0.10	3.1	4.6	3.8±0.14	3.0	4.9	6.0±0.09
Cysteine and/or cystine	1.1±0.00	1.3±0.07	1.3	1.1	0.6±0.08	1.3	1.6	1.3±0.00
Methionine	1.7±0.18	3.5±0.12	2.4	2.3	3.0±0.09	2.3	3.3	3.4±0.04
Total	104.8	105.1	109.8	103.5	105.0	106.2	106.4	104.5
Total calculated as amino acid residues/100 g. protein	91.2	91.2	95.2	89.7	90.5	90.60	92.1	92.7

* Abbreviations for host insects: *Pd*, *Porthetria dispar* (L.), gypsy moth; *Cf*, *Choristoneura fumiferana* (Clem.), spruce budworm (this insect is the host of both polyhedral and capsule type viruses: the inclusion protein of the former and the virus of the latter were analysed); *Ma*, *Malacosoma americanum* (F.), eastern tent caterpillar; *Md*, *Malacosoma disstria* Hbn., forest tent caterpillar; *Bm*, *Bombyx mori* L., silkworm; *Cpe*, *Colias philodice eurytheme* Bdv., alfalfa butterfly; *Ns*, *Neodiprion sertifer* (Geoffr.), pine sawfly; *Cm*, *Cacoecia murinana* Hb.

† Mean value and its standard error.

Table 2. *Amino acid contents of insect viruses*

Expressed as percentage of total recovered amino acids. The presence or absence of tryptophan could not be established.

No. of hydrolysates analysed ...	Polyhedral type*					Capsule type*	
	<i>Pd</i>	<i>Ma</i>	<i>Md</i>	<i>Bm</i>	<i>Cpe</i>	<i>Cm</i>	<i>Cf</i>
	2	2	1	2	2	1	2
Amino acid							
Aspartic acid	12.0	11.0	11.0	13.3	13.2	14.0	13.2
Glutamic acid	8.5	9.3	10.0	6.9	7.6	7.0	6.9
Histidine	0.9	1.5	1.1	0.7	1.2	1.9	1.6
Lysine	2.1	3.5	3.5	3.3	2.2	3.4	3.1
Arginine	19.8	16.1	15.5	11.1	19.5	16.4	16.3
Glycine	6.1	4.0	4.9	8.1	3.5	4.6	5.0
Alanine	5.9	3.9	3.6	4.5	3.5	3.6	3.5
Valine	4.4	3.6	3.1	3.8	3.7	3.6	3.9
Leucine and/or isoleucine	11.0	11.8	12.7	11.2	11.4	13.0	11.7
Proline	6.4	6.3	5.2	4.9	4.5	5.3	7.6
Phenylalanine	4.5	4.7	5.5	4.6	4.2	5.6	5.4
Tyrosine	2.8	4.8	6.1	6.2	6.3	5.0	4.7
Serine	9.4	10.5	9.0	8.5	9.2	9.8	9.2
Threonine	4.4	6.0	6.4	9.6	6.7	3.8	5.0
Cysteine and/or cystine	0.9	0.8	0.6	0.7	1.1	0.6	0.6
Methionine	1.0	2.4	2.4	2.8	1.8	2.8	2.4
g. Amino acid recovered/100 g. virus	57.5	87.1	83.7	75.9	77.4	—	—

* Abbreviations as in Table 1.

weight attributable to nucleic acid, there was no possibility of determining the percentage recovery of the amino acids with respect to virus protein. In addition, no correction was made for the glycine formed by hydrolysis of the nucleic acids.

The different viruses and proteins are arranged in the tables in the order of Wyatt's (1952) stepwise grouping based on the ratio of adenine plus thymine: guanine plus cytosine. *Pd* is alone in his first group, *Cf* (*Choristoneura fumiferana*) alone in the second, *Ma* (*Malacosoma americanum*), *Md* (*Malacosoma disstria*), *Bm* and *Cpe* (*Colias philodice eurytheme*) are in the fourth, *Ns* (*Neodiprion sertifer*) and *Cm* (*Cacoecia murinana*) in the fifth and the capsule type *Cf* in the sixth group.

Purified virus material was not available for analyses of viruses of the polyhedral diseases of *Cf* or *Ns*.

DISCUSSION

Inclusion-body proteins. It is evident from Table 1 that the proteins characteristic of insect virus inclusion bodies are very similar to one another in amino acid composition. Although many significant differences can be observed between them, these proteins seem to form a group with very similar properties.

From analogy with the work on immune proteins by Smith & Greene (1947) it was not unexpected that the inclusion-body proteins of the polyhedral diseases of insects *Pd*, *Cf*, *Ma*, *Md*, *Bm* and *Cpe* in Table 1 should be similar, since the inclusion bodies are all of the polyhedral type, and are formed in the nuclei of most of the body tissues of the diseased insects. However, in the case of the diseases of *Ns* and *Cm*, greater differences might be expected. For although the disease of *Ns* is characterized by polyhedral inclusion bodies appearing in the cell nuclei each containing many virus particles, these are restricted to the physiologically specialized cells of the gut wall. With that of *Cm*, the inclusion bodies are of very different shape, occlude only one virus particle each, and are found in the cytoplasm of the cells, yet the inclusion protein does not differ in composition from the others more than they differ among themselves.

If all the values in Table 1 are assumed to have standard errors similar to those which could be calculated, and the significance of differences examined by the Student's *t* test, from one to six significant differences ($P < 0.01$) can be found in comparing any two of these proteins.

Viruses. The results in Table 2 show that the insect viruses of both polyhedral and capsule types are closely related with respect to the relative proportions of the various amino acids which they contain. However, assuming standard errors comparable to those of the analyses of the inclusion-

body proteins, differences distinguishing one virus from another on the basis of their amino acid constituents do appear. These differences seem to follow the morphological classification (Bergold, 1953) rather than the groupings based on the nucleic acid analyses. For example, although Wyatt (1952) found that the two capsule viruses differ in the composition of their nucleic acid, these two morphologically very similar viruses have very similar amino acid composition. Conversely, the virus of *B. mori* L. and the two tent-caterpillar viruses (*Ma* and *Md*) could not be distinguished by nucleic acid composition but morphologically the *Bm* virus is quite different from the two similar tent-caterpillar viruses. Again, the amino acid analyses follow the morphological classification, with fewer and smaller differences separating the *Ma* and *Md* viruses as compared with the differences between either of these and the *Bm* virus.

The virus from *Porthetria dispar* (L.) falls into a separate group on the basis of its amino acids, nucleic acid, and morphology.

Comparison of amino acid composition of virus and inclusion-body protein

The amino acid composition of the insect viruses differs most markedly from that of their characteristic inclusion-body proteins in the relative proportions of arginine, serine, lysine and tyrosine. Proportional to the total amino acid content the viruses contain approximately twice as much arginine and serine and only one half as much lysine and tyrosine as the inclusion-body proteins.

An interesting difference in variability between the two groups is observable in that a comparison between any two viruses and their respective inclusion-body proteins shows that the viruses differ significantly in more amino acids than do the inclusion-body proteins. An extreme example of this is found in the comparison of *Pd* with *Md* in which there are twice as many significant differences between the two viruses as there are between their inclusion-body proteins. This would suggest that the inclusion-body proteins are a more closely related group than the proteins of the virus. This fact together with the very significant differences in quantitative composition of the two groups and the large excess of inclusion-body proteins produced during multiplication of these viruses (at least $20 \times$ the weight of the total virus) may lend credence to the view that the polyhedral protein is a host response to the virus (Bergold & Wellington, 1954).

SUMMARY

1. The amino acid composition of a number of insect viruses and their characteristic inclusion-body proteins has been determined by quantitative paper chromatography.

2. The seven viruses analysed all have a similar pattern of amino acid composition and this differs markedly from the pattern of the eight inclusion-body proteins. Proportional to the total amino acid content, the former have a strikingly greater content of arginine and serine, and less lysine and tyrosine than the latter.

3. There is more variability among the viruses than among the inclusion-body proteins, but significant differences exist between any two members of either group.

4. The differences in amino acid composition tend to parallel the morphological classification (Bergold, 1953) rather than the groupings based on the nucleic acid analysis (Wyatt, 1952).

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Amino Acid Utilization in Bacterial Growth

2. A STUDY OF THREONINE-ISOLEUCINE RELATIONSHIPS IN MUTANTS OF *ESCHERICHIA COLI*

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The possibility that threonine is an intermediate in the synthesis of isoleucine in micro-organisms has been considered in earlier publications (Teas, 1948; Umbarger & Adelberg, 1951). Teas (1948) described growth experiments in which isoleucine-requiring mutants of *Neurospora* were able to use the keto analogue, α -oxo- β -ethylbutyric acid, as well as any of a series of four-carbon compounds; namely, α -aminobutyric acid, α -oxobutyric acid and D-threonine. Evidence from growth studies for the relationship between threonine and isoleucine in micro-organisms has been summarized by Umbarger & Adelberg (1951). From their analysis of the data they have classified the threonine-requiring organisms into three classes: (1) those which respond to L-threonine only; (2) those which show an absolute requirement for isoleucine, D-threonine or

one of several other four-carbon compounds; (3) a group which responds as group 2 but will utilize L-threonine also.

The present paper is concerned principally with the relationship of L-threonine to isoleucine and to the four-carbon intermediates, D-threonine, α -aminobutyric acid, α -oxobutyric acid and α -oxo- β -hydroxybutyric acid. The approach has been to combine inhibition analysis (Shive, 1950) with quantitative growth measurement employing several mutants of *Escherichia coli* (Maas & Davis, 1950).

EXPERIMENTAL

Strains used. *Escherichia coli*, wild type ML3 (Monod), L-threonine requirers ML41, ML52 and ML54 obtained by Monod from the wild type ML3.

Esch. coli, wild type W (ATCC 9637) (Davis), L-threonine requirer M43-6 from wild type W, and D-threonine requirer M97-21 from wild type W, both obtained by Davis. All strains were maintained on meat-broth agar slants by weekly transfer.

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