The Isolation and Properties of the Protein Calliphorin

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1. A procedure for the isolation of the protein calliphorin from larvae and pupae of the blowfly *Calliphora erythrocephala* is described. 2. The calliphorin preparation shows a single component in the ultracentrifuge at pH 6.3 and gives a single band when stained for protein after agar-gel or starch-gel electrophoresis at pH 6.3 or 8.6. Immunoelectrophoresis yields only one arc, associated with the stained spot, to a rabbit antiserum known to react with 13 other soluble components of *Calliphora* pupae. 3. Calliphorin has $s_{20,w}^0$ 19.4 S, $D_{20,w}^0$ $3.25 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ and f/f_0 1.22, indicating a molecular weight of 528 000 and a compact symmetrical structure. The molecular weight determined by the meniscus-depletion sedimentation-equilibrium method is 529 000. 4. In 6.2 M-guanidine hydrochloride calliphorin dissociates into six components each with a molecular weight of about 87 000. Calliphorin reversibly dissociates into components with sedimentation coefficients of about 7S as the pH is raised progressively above pH 6.5. 5. Calliphorin has an unusually high tyrosine and phenylalanine content (442 and 400 mol/mol of protein respectively), a relatively high methionine content (162 mol/mol of protein) and very little cystine or cysteine (18 mol/mol of protein). The E_{280}/E_{250} ratio is 3.2. The pure protein contains 0.4–0.5% carbohydrate. 6. When examined in the electron microscope by the negative staining technique the protein is seen to consist of particles which are right prisms, being 105 Å wide and 65 Å high, rectangular in side view and curvilinear equilateral triangles in surface view.

Electron-microscopic examination by the negative staining technique has revealed the presence of large numbers of particles, about 100 Å across, in extracts of larval, pupal and newly emerged adult *Calliphora erythrocephala* (Insecta, Diptera). These particles have been shown to be a protein for which the name 'calliphorin' has been proposed (Munn, Feinstein & Greville, 1967). Calliphorin is synthesized by the larval fat-body (Munn, Price & Greville, 1969). It is first detected in 2-day-old larvae, and in 4-day-old larvae it accounts for nearly 60% of the extractable soluble material. In pupae and adults the amount of calliphorin progressively decreases (Munn & Greville, 1969).

Calliphorin occurs in insects closely related to *Calliphora*, and particles that appear similar to calliphorin in the electron microscope and/or of similar behaviour in the ultracentrifuge appear to be of widespread occurrence in other insects (Greville, Munn & Feinstein, 1967; Munn & Greville, 1969). Because of the quantitative importance of calliphorin to the insects and the economic importance of some of these, we have prepared the protein in a highly purified form and examined a number of its properties.

* Deceased December 1969.

MATERIALS AND METHODS

Materials. Water was redistilled in glass and A.R. chemicals were used wherever available.

Rearing method. The *C. erythrocephala* were kept at a temperature of 23–28°C. Adults were supplied with sucrose, Bacto yeast extract and water. Eggs were collected on fresh lean meat and the growing larvae were provided with more meat at intervals for 4–5 days and were then allowed to pupate in sawdust.

Isolation of calliphorin. Calliphorin was isolated from larvae and from pupae. The animals (200–600) were first cooled to 0°C and the isolation procedure was carried out at 0–4°C. Whole larvae and pupae with or without their puparia were crushed in a solution containing 0.13 M-NaCl–17 mM-phosphate buffer (KH_2PO_4 adjusted to pH 6.3 with NaOH)–10 mM- MgCl_2 –4 mM-HCN, pH 6.3. The resulting brei was strained through two layers of muslin and the fluid was centrifuged at 10 000 g_{av} for 15 min. The supernatant was centrifuged at 150 000 g_{av} for 1 h in the AH50 rotor of a Spinco model L centrifuge to sediment the ribosomes. In some preparations this pellet was washed once in the centrifuge to recover some of the calliphorin that it contained. The calliphorin was completely sedimented from the supernatant by centrifugation at 150 000 g_{av} for 4 h. It was washed once in the centrifuge and resuspended in 4–6 ml of the isolation medium. Up to 2 ml of the suspension was layered over a 28 ml linear 5–20% (w/v) sucrose density gradient

containing 0.15 M-NaCl-20 mM-sodium-potassium phosphate buffer, pH 6.3, and centrifuged in the SW25 rotor of the Spinco model L centrifuge for 9-10 h at 22500 rev./min (51500 $g_{av.}$). Each gradient was then fractionated into 18-20 approximately equal fractions by collecting drops from a hole made in the bottom of the tube. The E_{280}/E_{250} ratio of each fraction was determined and those fractions having a ratio of 2.8 or greater were pooled, diluted with 0.15 M-NaCl-20 mM-sodium-potassium phosphate buffer, pH 6.3, and centrifuged at 150000 $g_{av.}$ for 4 h to sediment the calliphorin. The pellet was resuspended in a small volume of 50 mM-sodium-potassium phosphate buffer, pH 6.3, and subjected to zone electrophoresis on Pevikon powder (Shandon Scientific Co. Ltd., London N.W.10, U.K.) in 50 mM-sodium-potassium phosphate buffer, pH 6.1, with 0.2 M-sodium-potassium phosphate buffer, pH 6.1, in the reservoirs. The Pevikon block was 25 cm $\times$ 20 cm and 1 cm thick. The sample was applied 19 cm from the anode and a potential difference of 3 V/cm was applied for about 36 h. Distribution of protein on the block was ascertained by examination of a test strip (see Fig. 3), and when sufficient separation had occurred the Pevikon was cut into 1 cm-wide strips and these were extracted with 0.13 M-NaCl-17 mM-sodium-potassium phosphate buffer, pH 6.3. At a late stage in these experiments it was discovered that up to 50% of the protein applied to the Pevikon could not be eluted, even by a variety of drastic procedures. When some of the calliphorin which had been eluted was reapplied to the Pevikon the same proportion again could not be recovered and there was no positive evidence that the calliphorin which could be eluted was different from that which could not. The extracts were examined by immunoelectrophoresis and their E_{280}/E_{250} ratios were determined. Those fractions containing only calliphorin (as judged by immunoelectrophoretic behaviour) were pooled and concentrated by centrifugation for 4 h at 150000 $g_{av.}$. The pellets were suspended in a small volume of 0.15 M-NaCl-20 mM-sodium-potassium phosphate buffer, pH 6.3, and dialysed overnight against two changes of 500 vol. of this solution. The preparations were stored frozen at -18°C .

Analytical centrifugation. This was carried out in a Beckman-Spinco model E ultracentrifuge equipped with RITC temperature control. Before centrifugation the samples were dialysed against 0.148 M-NaCl-20 mM-potassium phosphate buffer, pH 6.3, or 50 mM-NaCl-20 mM-sodium-potassium phosphate buffer, pH 6.3, and their concentrations were then determined with a Laboratory Interferometer (Carl Zeiss, Jena, Germany). The sedimentation and diffusion coefficients were determined over a range of concentrations and $s_{20,w}^0$ and $D_{20,w}^0$ values determined by extrapolation. The behaviour of the intact protein and subunits obtained in 6.2 M-guanidine-hydrochloride was also examined in the An J rotor by the meniscus-depletion sedimentation-equilibrium method (Yphantis, 1964).

Preparation of antisera. Antisera to extracts of pupae of *Calliphora* ('crude antigen preparation') and to pure calliphorin were obtained from rabbits as described by Munn & Greville (1969).

Immunoelectrophoresis. Electrophoresis was carried out in agar gel [1.5% (w/v) Ionagar no. 2 (Difco) in 25 mM-sodium-potassium phosphate buffer, pH 6.1, con-

taining 0.1% of sodium azide], with 50 mM-sodium-potassium phosphate buffer, pH 6.1, in the reservoirs, for 3-4 h at 6 V/cm. Immunodiffusion was carried out at room temperature.

Amino acid analyses. Portions of the protein were hydrolysed with constant-boiling glass-distilled HCl for 24, 48 and 72 h *in vacuo* at 105°C . The hydrolysates were evaporated to dryness over NaOH pellets in a vacuum desiccator, then taken up in 0.5 ml of 67 mM-sodium citrate buffer, pH 2.0. Samples were analysed on a Technicon micro-analyser (Technicon Instruments Co. Ltd., Chertsey, Surrey, U.K.) by the procedure of Beale & Kent (1968). Cysteine was determined after 10 mg portions of calliphorin, dissolved in 0.4 ml of 98% formic acid, had been oxidized with 0.4 ml of a mixture containing 95% formic acid-5% H_2O_2 (100 vol.) for 3 h at 4°C and then taken up in water and freeze-dried, before hydrolysis for 24 h as above. Tryptophan was determined by methods based on u.v. spectrophotometry (Goodwin & Morton, 1946; Beaven & Holiday, 1952; Bencze & Schmid, 1957).

Carbohydrate analysis. Carbohydrate was determined by the method of Winzler (1955).

Electron microscopy. Samples were applied to carboned celloidin films or holey-carbon films (Huxley & Zubay, 1960) on grids and excess of NaCl and phosphate (or, in the case of protein crystals, ammonium sulphate) were washed away with several drops of ice-cold negative stain (2% sodium phosphotungstate, pH 7.1, or 2-5% ammonium molybdate, pH 6.9). Excess of liquid was removed with filter paper and the grids were allowed to dry. They were examined in a Siemens Elmiskop or an AEI EM6B electron microscope.

RESULTS

Purity of calliphorin during isolation. Although calliphorin on a quantitative basis is the major soluble constituent of larvae and pupae, there are present in addition at least 13 other soluble components, all of which are probably proteins (Munn & Greville, 1969) and which are present in the supernatant after centrifugation at 10000 $g_{av.}$ for 15 min. The immunoelectrophoretic pattern given by this material is shown in Plate 1(a) and its sedimentation pattern in Fig. 1(a). Calliphorin has no known enzymic property, but we found by experience that it had an unusually high E_{280}/E_{250} ratio, that it formed a sharp peak of about 18S in the ultracentrifuge and its specific precipitin arc could easily be recognized after immunoelectrophoresis; these criteria, particularly the last, were used to identify the protein during its isolation. After removal of the ribosomal fraction the sedimented calliphorin preparation showed a sharp symmetrical peak with a sedimentation coefficient of about 18S in the analytical ultracentrifuge and some more slowly sedimenting material to the extent of 7% or less (Fig. 1b). The behaviour of this material on density-gradient centrifugation is shown in Fig. 2. The material concentrated

EXPLANATION OF PLATE I

Agar-gel electrophoretic patterns of calliphorin preparations at three stages during the purification procedure: (a) 10 000 g_{av} supernatant; (b) pooled material after density-gradient centrifugation; (c) pure calliphorin. In each case the plate stained for protein is shown on the right and the immunoelectrophoretic pattern on the left.

EXPLANATION OF PLATE 2

(a) and (b) Electron micrographs of negatively stained material: (a) NaCl-sodium-potassium phosphate extract of whole pupae; (b) pure calliphorin. The bar represents 500 Å. (a) and the inset in (b) were negatively stained with sodium phosphotungstate, (b) with ammonium molybdate.

Fig. 1. Sedimentation patterns of calliphorin preparations at three stages during the purification procedure: (a) 10000 g_{av} supernatant, photographed 56 min after reaching the speed of 42040 rev./min. The bar angle was 50°. (b) Washed 150000 g_{av} pellet suspension, photographed 24 min after reaching the speed of 59780 rev./min. The bar angle was 45°. (c) Pure calliphorin, photographed 36 min after reaching the speed of 42040 rev./min. The bar angle was 55°. Sedimentation from left to right.

Fig. 2. Purification of calliphorin: fractionation of washed 150000 g_{av} pellet suspension by density-gradient centrifugation. Protein solution (1–2 ml) in isolation medium was layered over a 28 ml 5–20% (w/v) sucrose gradient in 0.146 M-NaCl–20 mM-sodium–potassium phosphate buffer, pH 6.1. The gradient was centrifuged at 22500 rev./min in the SW rotor of a Spinco model L centrifuge for 9 h, then fractionated by collecting drops from a hole pierced in the bottom of the tube. $\circ$, E_{280} ; $\triangle$, E_{250} ; $\square$, E_{280}/E_{250} ratio.

from the pooled fractions having an E_{280}/E_{250} ratio greater than 2.8 showed only a single symmetrical peak in the analytical ultracentrifuge, but immunoelectrophoresis showed this to contain two components (Plate 1b). These two components were separated by zone electrophoresis on Pevikon powder (Fig. 3), and the fractions containing aelliphorin alone were pooled and the calliphorin was sedimented by centrifugation at 150000 g_{av} for 4 h.

The final product at a concentration of 1% gave a single band when stained for protein after agar-gel

or starch-gel electrophoresis at pH 6.3 or 8.6, and a single arc, associated with the stained spot, when the agar-gel plate was developed with a rabbit antiserum to the crude antigen preparation (Plate 1c). In the analytical ultracentrifuge the calliphorin showed a single symmetrical peak at pH 6.3 (Fig. 1c).

The molecules of calliphorin appear to form a smoothly changing series of electrophoretically different species in the same way as γ -globulin in mammalian serum; successive fractions taken from the calliphorin zone on Pevikon showed increasing mobilities when subjected to electrophoresis on agar gel (Fig. 3). The calliphorin in these fractions, when recombined, gave an elongated precipitin arc (Plate 1c) closely similar to that given by calliphorin in the starting material (Plate 1a), although it must have lacked the slowest-moving species as they were discarded owing to their contamination with another protein ('Protein II'; Munn & Greville, 1969).

Hydrodynamic properties and molecular weight. The sedimentation coefficient in 50 mM-sodium chloride–25 mM-sodium–potassium phosphate buffer, pH 6.3, is concentration-dependent (Fig. 4). Results were obtained for five separate preparations of the protein and these gave the regression line $s_{20,w} (S) = 19.44 - 1.567c$, where c is the concentration of calliphorin in g/100 ml. The s.e. of $s_{20,w}$ is 0.104S and the s.e. of the slope is 0.172.

The $D_{20,w}^0$ determined for three separate preparations of calliphorin was $3.26 \times 10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ (Fig. 5). The results fitted the regression line $10^7 \times D_{20,w} = 3.26 - 0.127c$. The s.e. of $D_{20,w}^0$ is 0.0315 and the s.e. of the slope is 0.0539.

The molecular weight computed from these results was 528000; the frictional ratio (f/f_0) was

Fig. 3. Final stage in the purification of calliphorin: zone electrophoresis on Pevikon powder. A sample containing the calliphorin was applied at the origin (0) and subjected to electrophoresis for about 36 h at a potential difference of 3V/cm. Strips of paper were applied edge-on to the block, dried and stained for protein; when these showed separation of the material into two bands (a) the Pevikon block was cut into strips 1 cm wide. The proteins were eluted with 0.13 M-NaCl-17 mM-sodium-potassium phosphate buffer, pH 6.3. The E_{280} of each fraction was measured (b) and portions of fractions containing the bulk of the protein were examined by immunoelectrophoresis (c).

calculated to be 1.216. It is inferred that the calliphorin molecule is fairly symmetrical and compact. The molecular weight was determined indepen-

dently by the meniscus-depletion sedimentation-equilibrium method. The values obtained from three runs in NaCl-sodium-potassium phosphate

buffer, pH 6.3 and *I* 0.1, at a protein concentration of 0.05% at 5563, 6569 and 7928 rev./min in the An J rotor were 546000, 519000 and 523600 respectively, giving a mean value of 529000.

The protein dissociated into subunits when dialysed against 6.2M-guanidine hydrochloride, pH 8.0. The molecular weight of the subunits obtained in the presence of 40 mM-iodoacetamide was

87470 at a protein concentration of 0.056% and 85620 at protein concentration of 0.028%. In one experiment in which the calliphorin was both reduced and blocked the molecular weight of the subunits was found to be 72000. It was concluded therefore that there are six or possibly seven subunits in the molecule and that there are no inter-chain disulphide bridges.

As the pH is progressively raised above 6.3 (in NaCl-sodium-potassium phosphate buffer, *I* 0.1)

Fig. 4. Concentration-dependence of the sedimentation coefficient of calliphorin in 50 mM-NaCl-33 mM-sodium-potassium phosphate buffer, pH 6.3. The results were obtained from five different preparations denoted by the different symbols.

Fig. 5. Concentration-dependence of the diffusion coefficient of calliphorin in 50 mM-NaCl-33 mM-sodium-potassium phosphate buffer, pH 6.3. The results were obtained from three different preparations denoted by the three types of symbol.

Fig. 6. Sedimentation patterns showing reversible dissociation of calliphorin at pH 8.6. Samples were dialysed against sodium veronal buffer, pH 8.6 and *I* 0.1, for: (a) 4 h; (b) and (c) 22 h; (d) 9 days. A different preparation was dialysed at pH 8.6 for 24 h (e) and then back to pH 6.3 for 24 h (f). The photographs were taken 16 min [(a), (b), (d) and (e)], 64 min (c), and 32 min (f) after reaching the speed of 59780 rev./min [(a)-(d)] and 52000 rev./min [(e) and (f)]. The bar angle was 50°. Sedimentation was from left to right.

Table 1. *Amino acid composition of calliphorin*

	Amino acid (mol/mol of protein of mol.wt. 530 000)				
	Oxidized and hydrolysed (24 h)	Hydrolysed			Averaged or extrapolated*
		72 h	48 h	24 h	
Cysteic acid	17	—	—	—	18
Aspartic acid	416	426	435	420	426
Threonine	310	154	160	167	172*
Methionine					
sulphone					
Serine	144	122	131	143	154*
Glutamic acid	326	351	365	367	361
Proline	114	109	108	104	110
Glycine	193	195	193	191	193
Alanine	120	114	117	121	117
Valine	196	213	209	206	213†
Methionine	—	120	131	149	162*
Isoleucine	137	146	140	142	143
Leucine	204	211	206	210	209
Tyrosine	247	346	368	410	442*
Phenylalanine	384	401	387	408	400
Lysine	291	295	291	300	295
Histidine	127	122	115	117	118
Arginine	127	116	107	126	116
Tryptophan	—	—	—	—	64‡

* Extrapolated to zero time.

† 72 h hydrolysis value.

‡ Determined separately.

calliphorin increasingly dissociates into units with a sedimentation coefficient of about 7S. In samples dialysed for 24h at pH 7.8 some 10% of the calliphorin dissociates. At pH 8.6 (sodium veronal buffer, 10.1) the dissociation is much more extensive. After dialysis for 4h 73% of the calliphorin has dissociated (Fig. 6a) and after 22h 90% is dissociated (Fig. 6b). The dissociated material does not give a symmetrical peak in the ultracentrifuge (Fig. 6c). Prolonged periods of dialysis increase the amount of more slowly sedimenting units only slightly; thus after 9 days at pH 8.6 7% of the calliphorin remains intact (Fig. 6d). The dissociation is reversible; material dialysed for 24h at pH 8.6, then dialysed against sodium-potassium phosphate-NaCl buffer, pH 6.3, for a further 24h is all restored to the 19S form (Figs. 6e and 6f).

Amino acid composition. The amino acid compositions of samples of calliphorin that had been oxidized, and/or hydrolysed for 24, 48 and 72h and the averaged or extrapolated zero-time values are shown in Table 1. The noteworthy features of the amino acid composition are the unusually high content of the aromatic amino acids tyrosine and phenylalanine and the relative paucity of half-cystine residues. Assuming six subunits/molecule of calliphorin, each subunit will contain 3 residues

of half-cystine, 74 residues of tyrosine, 67 residues of phenylalanine, 71 and 60 residues respectively of aspartic acid and glutamic acid and smaller amounts of the other amino acids, most notable of which is perhaps the 27 residues of methionine (Table 1).

Absorption spectrum. The u.v.-absorption spectrum of calliphorin in 0.148M-NaCl-20mM-sodium-potassium phosphate buffer, pH 6.3, has an unusually high E_{280}/E_{250} ratio. Values for different preparations range from 3.01 to 3.53 with a mean value of 3.2. The $E_{\max}$ was at 278nm and the $E_{\min}$ at 250nm.

Carbohydrate content. Pure calliphorin gives a weak positive reaction when stained for carbohydrate by the periodate-formazan reaction (Munn & Greville, 1969). Quantitative analyses with mannose and galactose as standards indicated that the carbohydrate content was 0.4-0.5%.

Electron-microscopic appearance. The appearance of the negatively stained protein in a NaCl-sodium-potassium phosphate extract of whole pupae is shown in Plate 2(a) and the pure protein, at considerably higher magnification, is shown in Plate 2(b). The particles appear to be right prisms, being 105 Å wide and 65 Å high, rectangular in side view and curvilinear equilateral triangles in surface view (Fig. 7). In some of the electron micrographs

Fig. 7. Shape and dimensions of calliphorin molecule deduced from examination of negatively stained preparations in the electron microscope. The broken lines show the possible shape of six identical self-assembling subunits. (a) is arbitrarily called the 'plan' view and (b) is the 'side' view.

there is evidence of substructure in the intact calliphorin molecules (inset, Plate 2b).

DISCUSSION

The isolation procedure described is relatively simple, will yield up to 200mg of pure calliphorin and can be completed within 1 week. As noted in the Materials and Methods section, the final purification step involving zone electrophoresis on Pevikon powder is rather wasteful because of the difficulty of recovering calliphorin from the Pevikon. Future work might more profitably use an alternative stationary phase for the preparative electrophoresis.

The properties of the intact calliphorin molecule deduced from its behaviour in the ultracentrifuge, namely molecular weight of 530 000 and compact symmetrical structure, are in good agreement with its appearance in the electron microscope, although the molecular weight calculated from the shape and dimensions of the negatively stained protein, assuming a density of 1.2, is only 480 000. The relationship between the substructure sometimes seen in the molecules in the electron microscope and the subunits obtained by treatment with high pH and/or with guanidine hydrochloride is not clear. The subunits produced at pH 8.6 have a sedimentation coefficient of about 7S, but it is not possible to calculate their molecular weight because they are clearly not homogeneous (Fig. 6c). It seems not unreasonable, however, to equate the

six subunits produced irreversibly in guanidine hydrochloride with those obtained reversibly at pH values above pH 6.5. One way in which six identical subunits could form the shape deduced for the molecule from its appearance in the electron microscope is shown in Fig. 7.

The unusually high E_{280}/E_{250} ratio of calliphorin is presumably due to the relatively high proportion of tyrosine that the protein contains. However, disaggregation of the subunits as the pH is raised is unlikely to be related to the ionization of the tyrosine residues, since there is very little change in the E_{295} at pH 8.6 compared with that at pH 6.1. Calliphorin appears to have the highest proportion of tyrosine of any protein whose amino acid composition is known and the proportion of phenylalanine is also high (Table 1). Papain, with 14.7% tyrosine, is fairly similar in this respect to calliphorin, but has only 3.1% phenylalanine (Smith, Stockell & Kimmel, 1954). Similarly silk fibroin from *Bombyx mori*, with 12% tyrosine, contains only 1% phenylalanine (Lucas, Shaw & Smith, 1960). Insulin, with 12.4% tyrosine and 8.5% phenylalanine, and glucagon, with 10.6% tyrosine and 9.4% phenylalanine, are the only proteins with a total aromatic amino acid content approaching that of calliphorin (Harfenist, 1953; Behrens & Bromer, 1958).

Attempts to identify the N-terminal residues were inconclusive, partly because of the formation of large amounts of DNS-tyrosine and partly because the protein became insoluble when treated with urea, a property that calliphorin shares with the seed protein arachin (Johnson & Shooter, 1950).

It has been proposed that calliphorin, like the seed proteins, functions as a store for amino acids that is laid down by the larval form of the insect and utilized for the formation of the adult tissues (Greville *et al.* 1967; Munn & Greville, 1969). In the larva the pH of the haemolymph is about 6, but this increases to about 7, at a time when calliphorin begins to be utilized. Thus there may be some physiological significance in the dissociation of the protein at pH values above about 6.5. If calliphorin does function as a storage form for amino acids there must presumably be another one providing cysteine.

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REFERENCES

- Beale, D. & Kent, C. M. (1968). *Proc. 6th Technicon Amino Acid Colloquium, London*, p. 133. Chertsey: Technicon Instruments Co. Ltd.

- Beaven, G. H. & Holiday, E. R. (1952). *Adv. Protein Chem.* **7**, 319.
- Behrens, O. K. & Bromer, W. W. (1958). *Vitamins Horm.* **16**, 263.
- Bencze, W. L. & Schmid, K. (1957). *Analyt. Chem.* **29**, 1193.
- Goodwin, T. W. & Morton, R. A. (1946). *Biochem. J.* **40**, 628.
- Greville, G. D., Munn, E. A. & Feinstein, A. (1967). *Abstr. 7th int. Cong. Biochem., Tokyo*, p. 589.
- Harfenist, E. J. (1953). *J. Am. chem. Soc.* **75**, 5528.
- Huxley, H. E. & Zubay, G. (1960). *J. molec. Biol.* **2**, 10.
- Johnson, P. & Shooter, E. M. (1950). *Biochim. biophys. Acta*, **5**, 361.
- Lucas, F., Shaw, J. T. B. & Smith, S. G. (1960). *J. molec. Biol.* **2**, 239.
- Munn, E. A., Feinstein, A. & Greville, G. D. (1967). *Biochem. J.* **102**, 5.
- Munn, E. A. & Greville, G. D. (1969). *J. Insect Physiol.* **15**, 1935.
- Munn, E. A., Price, G. M. & Greville, G. D. (1969). *J. Insect Physiol.* **15**, 1601.
- Smith, E. L., Stockell, A. & Kimmel, J. R. (1954). *J. biol. Chem.* **207**, 551.
- Winzler, R. J. (1955). In *Methods of Biochemical Analysis*, vol. 2, p. 279. Ed. by Glick, D. New York: Interscience Publishers Inc.
- Yphantis, D. A. (1964). *Biochemistry, Easton*, **3**, 297.