Amino Acid Sequences around the Cystine Residues in Horse Growth Hormone

By LOUISE OLIVER AND ANNE STOCKELL HARTREE

Department of Biochemistry, University of Cambridge

(Received 15 February 1968)

The cystine-containing peptides of horse growth hormone were isolated and their amino acid sequences determined. Four unique half-cystine residues occur in two peptides, one containing 11 and the other, at the *C*-terminus of the protein, 15 amino acids. These sequences are compared with published data on growth hormones from other species.

The wide interest in recent years in the biochemistry of growth hormones has led to the determination of the complete amino acid sequence of human growth hormone (Li, Liu & Dixon, 1966) and of the *C*-terminal sequences in ox (Santomé, Wolfenstein & Paladini, 1965*a,b*; Santomé, Wolfenstein, Biscoglio & Paladini, 1966; Wallis, 1966) and pig (Mills, 1967) growth hormones. In each of these proteins, half-cystine is the third residue from the *C*-terminus of the polypeptide chain. We decided to compare another species, and we used the method of Brown & Hartley (1963, 1966) to isolate the cystine-containing peptides of horse growth hormone and determined their amino acid sequences. The *C*-terminal octapeptide of horse growth hormone is identical with that of pig growth hormone (Mills, 1967) and the adjacent pentapeptide is identical with that of ox growth hormone (Santomé *et al.* 1966). Except for the *C*-terminal octapeptide there is very little similarity in the sequences around the cystine residues of horse and human (Li *et al.* 1966) growth hormones.

MATERIALS AND METHODS

Horse growth hormone. This was prepared by the method of Raben (1959), or by alkaline extraction followed by DEAE-cellulose chromatography. The biological activities of the preparations ranged from 0.44 to 0.75 i.u./mg. (Hartree, Mills, Welch & Thomas, 1968). Amino acid analyses and peptide 'maps' of enzymic digests were identical for the different hormone preparations used in this study.

Human growth hormone. This was prepared by the method of Raben (1959) and further purified by IRC-50 chromatography (Hartree, Kovačić & Thomas, 1965) to a potency of 21 i.u./mg.

Digestive enzymes. Bovine 1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one-treated trypsin, α -chymotrypsin 1, papain, porcine pepsin and di-isopropyl phosphorofluoridate-treated carboxypeptidase A were

crystalline products from Worthington Biochemical Corp., Freehold, N.J., U.S.A., and subtilisin was obtained from British Drug Houses Ltd., Poole, Dorset.

Aminoethylation of growth hormone. This was performed by the method of Raftery & Cole (1963) as modified by R. N. Perham (personal communication). Growth hormone (50 mg.) was dissolved in 10 ml. of 1 M-tris at pH 8.5 containing 8 M-urea. 2-Mercaptoethanol (50 μ l.) was added and the solution was left at room temperature for 3 hr. Then 2 ml. of 1 M-tris at pH 8.5 and 0.4 ml. of ethyleneimine were added, and after 2½ hr. the mixture was dialysed for 40 hr. against glass-distilled water with repeated changes and freeze-dried. No cystine was detected on quantitative amino acid analysis of the product.

Enzymic digestion of growth hormone. Digestion with pepsin was performed at pH 2.0 at 37° for 1½–2 hr. in 5% (v/v) formic acid with 50–100 mg. of growth hormone and a 1:50 weight ratio of pepsin to growth hormone. Digestion with trypsin was at 37° for 4 hr. in 0.5% NH_4HCO_3 with a 1:50 weight ratio of trypsin to growth hormone.

Enzymic digestion of peptic peptides. For digestion with trypsin, chymotrypsin or subtilisin 0.1 μ mole of peptide was dissolved in 0.2 ml. of 0.5% NH_4HCO_3 at pH 8.0, and incubated at 37° for 2 hr. with 5 μ g. of enzyme. For papain digestion, 0.1 μ mole of peptide was dissolved in 0.2 ml. of pH 6.5 buffer (pyridine-acetic acid-water, 25:1:225, by vol.) and incubated at 37° for 4 hr. with 1 μ l. of 2-mercaptoethanol and 10 μ g. of papain. Carboxypeptidase A digestion was performed exactly as described by Ambler (1963).

Diagonal paper electrophoresis for cystine peptides. Groups of cysteine acid peptides were isolated from the peptide digest by the method of Brown & Hartley (1966).

Purification of peptides. After initial isolation of cysteine acid peptides by paper electrophoresis at pH 6.5, they were further purified by electrophoresis at pH 3.5 and at pH 2.0 and by descending paper chromatography (Waley & Watson, 1953). High-voltage paper electrophoresis was performed by the method described by Ryle, Sanger, Smith & Kitai (1955) in an apparatus similar to that of Michl (1951). The buffer systems and coolants at pH 6.5, 3.5 and 2.0 were as described by Ambler (1963). Peptides from tryptic digests of aminoethylated protein were isolated by descending paper chromatography (Waley & Watson, 1953) and further purified by electrophoresis at

pH 6.5 and at pH 3.5. For peptides that were difficult to purify, electrophoresis at pH 2.0 was also performed. The sulphur-containing peptides, were located by treatment with platonic iodide solution (Toennies & Kolb, 1951).

Amino acid analysis. For protein analyses samples (0.5 μ mole) were hydrolysed in constant-boiling HCl (6N) in sealed evacuated tubes at 105° for 24, 48, 72 and 96 hr. Additional samples were oxidized with performic acid (Hirs, 1956) and hydrolysed for 24 and 48 hr.

For analysis of peptides, samples (approx. 0.3 μ mole) were hydrolysed under similar conditions for 24 hr. A Technicon Auto-Analyser was employed for quantitative analysis of the hydrolysates. When the amount of hydrolysate available was too small (0.01–0.1 μ mole) for quantitative analysis, a qualitative estimation was obtained by paper electrophoresis at pH 2.0 and comparison with a standard mixture of amino acids.

Determination of N-terminal amino acids. For determination on the intact protein 10 μ moles of protein were dissolved in 0.5 ml. of 0.5M-NaHCO₃ containing 8M-urea and 0.5 ml. of DNS* chloride (containing 2mg./ml.) was added. The solution was left overnight at room temperature, and then dialysed against glass-distilled water for 48 hr. and freeze-dried. After hydrolysis with 6N-HCl for 16–24 hr. at 105°, the product was subjected to electrophoresis at pH 4.4 on a flat-plate apparatus (Shandon Scientific Co. Ltd., London, N.W. 10) for identification of the fluorescent DNS-amino acid (Gray & Hartley, 1963a).

For determination on peptides, 10 μ moles were allowed to react with DNS chloride for 2 hr. by the method of Gray (1967) as modified by Ambler & Brown (1967).

Amino acid sequence determinations. These were determined by the 'dansyl'-Edman technique (Gray & Hartley, 1963b; Gray, 1967).

RESULTS

Amino acid composition and terminal residues of horse growth hormone. The amino acid analyses on ten samples of hydrolysed protein were calculated as moles/mole of alanine and the mean $\pm$ s.d. for each amino acid is given in Table 1. Similar average molar ratios were obtained when the results were calculated in terms of moles/mole of leucine. Assuming integral molar ratios of the amino acid residues, there are 4 half-cystine and 3 methionine residues/mol., or some multiple thereof. The analysis of a 20 hr. hydrolysate of horse growth hormone reported by Saxena & Henneman (1966) is in good agreement with ours except that they found somewhat less methionine and more lysine and arginine.

Determination of N-terminal amino acids on several different preparations of horse growth hormone yielded only one N-terminal residue, phenylalanine. Digestion with carboxypeptidase overnight liberated only phenylalanine, but addition

* Abbreviations: DNS, 1-dimethylaminonaphthalene-5-sulphonyl; in amino acid sequences: CySO₃H, cysteic acid; MetSO₂, methionine sulphone; Asx, aspartic acid or asparagine.

Table 1. *Amino acid analysis of horse growth hormone*

The analytical results are the mean of six analyses on 24 hr., 48 hr., 72 hr. and 96 hr. hydrolysates of horse growth hormone plus three analyses on 24 hr. and 48 hr. hydrolysates of performic acid-oxidized protein and one analysis on a 24 hr. hydrolysate of aminoethylated protein. Each analysis was expressed as moles/mole of alanine so that the results could be pooled and averaged. Threonine recovery was constant for all hydrolysis times except 96 hr., and serine was unchanged except for the 72 hr. and 96 hr. hydrolysates, so these three values are not included. Valine and isoleucine recoveries did not increase with time of hydrolysis and so all values were averaged. A low tyrosine recovery in the 48 hr. hydrolysate of oxidized protein was not included. Methionine sulphone and aspartic acid were not separated and so were not determined in analyses on oxidized protein. The half-cystine analyses included are of oxidized or aminoethylated protein only. The mean ratios were calculated by assuming integral molar ratios of the eight amino acids present in lowest concentration. For comparative purposes the data of Saxena & Henneman (1966) were normalized to the same total number of residues as ours (excluding half-cystine and tryptophan).

Amino acid	Analysis (moles/mole of alanine)	Mean ratio	Ratio (results of Saxena & Henneman, 1966)
CyS	0.284 $\pm$ 0.020	3.9 $\pm$ 0.3	—
Asp	0.989 $\pm$ 0.056	13.7 $\pm$ 0.8	14.3
Thr	0.518 $\pm$ 0.062	7.2 $\pm$ 0.9	6.9
Ser	0.898 $\pm$ 0.053	12.5 $\pm$ 0.7	13.1
Glu	1.538 $\pm$ 0.138	21.4 $\pm$ 1.9	20.6
Pro	0.509 $\pm$ 0.024	7.1 $\pm$ 0.3	6.9
Gly	0.625 $\pm$ 0.042	8.7 $\pm$ 0.6	8.5
Ala	1.00	13.9	14.7
Val	0.531 $\pm$ 0.052	7.4 $\pm$ 0.7	6.7
Met	0.225 $\pm$ 0.019	3.1 $\pm$ 0.3	2.2
Ile	0.424 $\pm$ 0.058	5.9 $\pm$ 0.8	4.9
Leu	1.541 $\pm$ 0.104	21.4 $\pm$ 1.4	20.9
Tyr	0.431 $\pm$ 0.047	6.0 $\pm$ 0.6	4.7
Phe	0.676 $\pm$ 0.030	9.4 $\pm$ 0.4	9.0
Lys	0.605 $\pm$ 0.093	8.4 $\pm$ 0.7	10.7
His	0.187 $\pm$ 0.043	2.6 $\pm$ 0.6	2.8
Arg	0.815 $\pm$ 0.066	11.3 $\pm$ 0.9	13.2
Trp	—	—	1.6

of sodium dodecyl sulphate before digestion (Santomé *et al.* 1966) resulted in liberation of traces of alanine also. The presence of phenylalanine in both N-terminal and C-terminal positions is in agreement with the findings of Saxena & Henneman (1966). The release of traces of alanine in addition to phenylalanine by digestion with carboxypeptidase suggests that the C-terminal sequence is -Ala-Phe.

Cystine peptides in a peptic digest of horse growth hormone. The characteristic pH 6.5/pH 6.5 'diagonal map' of cysteic acid peptides in a peptic digest of

horse growth hormone is shown in Fig. 1. No cysteic acid peptides were found in the acidic area of the first-dimensional pH 6.5 electrophoresis. The peptides from bands A, B and C were further purified by paper electrophoresis at pH 3.5 and at pH 2.0. Peptides A1, A2 and B were then considered pure, since each had a single *N*-terminal amino acid and integral ratios of amino acid residues. The yields of peptides A1 and B from 15mg. of protein were 0.072 μ mole and 0.053 μ mole respectively. Yields of peptide A2 were very much smaller. The peptides from band C were obtained in very low yield and were not sufficiently pure for sequence

analysis. Data on mobilities and amino acid composition of the peptides are summarized in Table 2. Peptides in band C appeared to be mixtures of very large overlapping peptides containing the same cysteic acid residues as peptides from bands A and B. The amino acid composition of peptide C3 is given as an example.

Peptide A2 has the same amino acid composition as peptide A1 except for one less residue of valine and one less of arginine, and is likely to have arisen by peptic cleavage of peptide A1. Peptides A1 and B each contain two cysteic acid residues and are each assumed to have originally contained an internal disulphide bridge, since no other peptide appears off the diagonal in a vertical line with either. Peptide A2 presumably contains the same disulphide bridge as peptide A1. Peptides A1 and B were subjected to detailed sequence analysis.

Sequence studies of peptide A1. The sequence of the first five residues of the peptide determined by the 'dansyl'-Edman method was Arg-Val-MetSO₂-Lys-CySO₃H-. Peptide A1 was digested with trypsin, the resulting peptides were separated by electrophoresis at pH 6.5, and the sequence of each was determined by the 'dansyl'-Edman method. The results are summarized in Table 3. Peptide T1, which contains all the phenylalanine of peptide A1, is assumed to be *C*-terminal since the *C*-terminal residue of peptide A1 is phenylalanine (Table 2). The mobilities at pH 6.5 of peptides A1 and T1 suggest that peptide A1 has a net positive charge of 1 and peptide T1 a net negative charge of 2 (Offord, 1966), and therefore that the glutamic acid residue is not amidated. From the *N*-terminal sequence of five residues, the tryptic peptides were put in order, and with the *C*-terminal residue known (Table 2) the following sequence for peptide A1 is suggested: Arg-Val-MetSO₂-Lys-CySO₃H-Arg-Arg-Phe-Val-Glu-(Ser,Ser,CySO₃H,Ala)-Phe. Digestion of

Fig. 1. 'Cystine diagonal map' of horse growth hormone. Electrophoresis was at pH 6.5 in both dimensions. The major cysteic acid peptides are shaded. Histidine, neutral amino acids and the dye Xylene Cyanol FF (CyFF) were present as markers in the second electrophoresis, and are also shaded.

Table 2. *Cysteic acid peptides from a peptic digest of horse growth hormone*

Compositions are expressed as uncorrected ratios of amino acids after 24 hr. hydrolysis. Mobilities of peptides at pH 6.5 are given relative to aspartic acid = -1. Aspartic acid and methionine sulphone were not separated and both were present in the peptides derived from band C.

Peptide	Mobility at pH 6.5	Composition	Remarks
A1	+0.206	CySO ₃ H (1.8), MetSO ₂ (1.0), Ser (1.8), Glu (1.0), Ala (1.1), Val (1.9), Phe (1.9), Lys (1.1), Arg (2.9)	<i>N</i> -Terminal Arg, <i>C</i> -Terminal Phe
A2	0.00	CySO ₃ H (1.9), MetSO ₂ (1.1), Ser (1.9), Glu (1.2), Ala (1.1), Val (1.1), Phe (2.0), Lys (1.1), Arg (2.1)	<i>N</i> -Terminal MetSO ₂
B	-0.24	CySO ₃ H (2.0), Asp (2.0), Ser (0.9), Val (0.8), Ile (0.7), Leu (0.9), Tyr (0.5), Lys (1.0), Arg (1.0)	<i>N</i> -Terminal Leu, <i>C</i> -Terminal residue not released by carboxypeptidase A
C3		CySO ₃ H (1.2), Asp (+), MetSO ₂ (3.4), Thr (1.7), Ser (1.9), Glu (4.8), Pro (2.9), Gly (1.4), Ala (2.7), Ile (1.0), Leu (1.0), Phe (1.8), Lys (1.0), Arg (1.0)	<i>N</i> -Terminal Phe and Ala, impure

Table 3. *Amino acid sequences in the tryptic peptides derived from peptide A1*

Mobilities of peptides at pH 6.5 are relative to aspartic acid = -1.

Peptide	Mobility at pH 6.5	Composition and sequence
T1	-0.56	Phe-Val-Glu-(Ser,Ser,Ala,CySO ₃ H,Phe)
T2	0.00	CySO ₃ H-Arg
T3	+0.306	CySO ₃ H-Arg-Arg
T4	+0.53	Val-MetSO ₂ -Lys
T5		Arg

peptide A1 with papain yielded a peptide that contained serine, cysteic acid, alanine and phenylalanine and the sequence of the first three residues from the *N*-terminus determined by the 'dansyl'-Edman method was Ser-Ser-CySO₃H-. The complete sequence of peptide A1 can thus be deduced to be: Arg-Val-MetSO₂-Lys-CySO₃H-Arg-Arg-Phe-Val-Glu-Ser-Ser-CySO₃H-Ala-Phe. Further confirmation of the sequence was obtained by qualitative determination by paper electrophoresis at pH 2 of the amino acid compositions of peptides isolated from a papain digest and a subtilisin digest of peptide A1. Papain peptides, which contained (Arg,Val,MetSO₂), (Lys,CySO₃H,Arg,Phe,Val,Glu), (CySO₃H,Arg,Lys) and (Phe,Val,Glu,Ser,CySO₃H,Ala), and subtilisin peptides, which contained (CySO₃H,Ala,Phe), (Arg,Val,Met,Lys,CySO₃H), (Phe,Val,Glu,Ser), (Phe,Arg), (Arg,Lys,CySO₃H) and (Val,Glu,Ser,CySO₃H,Ala,Phe), were obtained, and are all consistent with the proposed sequence. The proposed sequence is also in agreement with formation of peptide A2 by peptic cleavage of the Val-Met bond of peptide A1.

Sequence studies of peptide B. The *N*-terminal sequence of the peptide determined by the 'dansyl'-Edman method was: Leu-Lys-CySO₃H-Arg-Ile-Val-. Tryptic digestion of peptide B followed by electrophoresis at pH 6.5 yielded four peptides; two of these were the expected dipeptides Leu-Lys and CySO₃H-Arg, and the sequence of the third was found to be Leu-Lys-CySO₃H-Arg. The fourth tryptic peptide was the *C*-terminal heptapeptide of peptide B. The electrophoretic mobility of peptide B at pH 6.5 suggested a net negative charge of 1 (Offord, 1966), and thus that one of the aspartic acid residues was amidated. Determination of the amino acid sequence of the tryptic heptapeptide by the 'dansyl'-Edman method was carried out until one of the aspartic acid residues had been removed and the sequence was determined as Ile-Val-Tyr-Asx-. A portion of the remaining tripeptide was allowed to react with DNS chloride and subjected to electrophoresis at pH 6.5 on the flat-bed apparatus. Its mobility with respect to aspartic acid was -0.49, suggesting a net negative charge of 1

(Offord, 1966) and that the remaining residue of aspartic acid was amidated. Three more Edman degradation steps with determination of the *N*-terminal residue by the 'dansyl' method after each step yielded DNS-aspartic acid, followed by DNS-aspartic acid plus DNS-cysteic acid and finally a trace of DNS-serine. The failure of carboxypeptidase A to liberate a *C*-terminal amino acid is explained by the presence of cysteic acid next to the *C*-terminal serine. The sequence of peptide B is thus deduced to be: Leu-Lys-CySO₃H-Arg-Ile-Val-Tyr-Asp-Asn-CySO₃H-Ser. Further confirmation of the proposed sequence was obtained from qualitative determination of the amino acid composition of peptides obtained from a chymotryptic digest and a subtilisin digest of peptide B. Chymotryptic peptides, which contained (Asp,CySO₃H,Ser) and (Leu,Lys,CySO₃H,Arg,Ile,Val,Tyr), and subtilisin peptides, which contained (Asp,CySO₃H,Ser) and (CySO₃H,Ser), are all consistent with the proposed sequence.

DISCUSSION

The amino acid composition and *N*- and *C*-terminal amino acids of our preparation agree well with those reported for the preparation of Saxena & Henneman (1966). The finding of integral molar ratios of the amino acids present in lowest concentration and a single residue in the *N*-terminal and *C*-terminal positions suggests that the hormone is sufficiently pure for detailed studies of its sequence.

The sequence of the *C*-terminal octapeptide of peptide A1 is identical with the *C*-terminal sequence of pig growth hormone (Mills, 1967) and shows several points of similarity with the corresponding sequences of ox (Wallis, 1966; Santomé *et al.* 1966) and of human (Li *et al.* 1966) growth hormones. Peptide A1 also contains at its *C*-terminus phenylalanine followed by alanine, the same amino acids that are released from the whole protein by carboxypeptidase. Therefore peptide A1 is very likely to be derived from the *C*-terminus of horse growth hormone.

A comparison of the *C*-terminal portions of

Table 4. *C-Terminal amino acid sequences of horse, ox and human growth hormones*

E, Horse growth hormone (the present work); B, ox growth hormone (Santomé *et al.* 1966); H, human growth hormone (Li *et al.* 1966).

E:	-Arg-Val-Met-Lys-CyS-Arg-Arg-Phe-Val-Glu-Ser-Ser-CyS-Ala-Phe

B:	-Met-Lys-CyS-Arg-Arg-Phe-Gly-Glu-Ala-Ser-CyS-Ala-Phe

H:	-Arg-Ile-Val-Gln-CyS-Arg-Ser-Val-Glu-Gly-Ser-CyS-Gly-Phe

horse, ox and human growth hormones (Table 4) shows that the sequence of 13 amino acids at the *C*-terminus of horse and ox growth hormones is identical except for two residues. There are more differences in the corresponding sequence of human growth hormone. Deletion of an arginine residue in the human hormone results in a disulphide ring one residue smaller.

The amino acid sequence of peptide B from horse growth hormone is very different from the sequences around the half-cystine residues of human growth hormone. In the latter protein the half-cystine residues nearest the *N*-terminus are separated by 93 amino acid residues, but in horse growth hormone only six residues are between the two half-cystine residues. There is no obvious similarity in the sequences around either half-cystine residue of the human protein with the sequence of peptide B. The possibility that peptide B was a contaminant in the preparation and not part of the horse growth hormone molecule seems unlikely, since it was found in all the preparations studied and was isolated in a yield approximately equal to that of peptide A1.

To make certain that the differences observed between human and horse growth hormones did not arise as a result of our methodology, we used the 'diagonal' technique of Brown & Hartley (1966) to isolate cysteic acid peptides from a peptic digest of human growth hormone. The amino acid analyses and *N*-terminal amino acids of the three peptides obtained were consistent with the sequence proposed by Li *et al.* (1966) and are assumed to be derived from residues numbered 68–80, 174–188 and 179–188 of their published sequence. In addition, the aminoethylated derivative of human growth hormone was digested with trypsin, three peptides containing *S*-aminoethylcysteine were isolated and the amino acid composition and *N*-terminal residue of each were determined. These results were also in agreement with the published sequence and the peptides corresponded to the residues numbered 57–68, 176–180 and 181–186 by Li *et al.* (1966).

Immunological studies of horse growth hormone

by the method of haemagglutination inhibition also suggest that ox and horse growth hormones have similar structures but differ considerably from human growth hormone. There is a strong cross-reaction between horse and ox growth hormones, and a very weak cross-reaction between horse and human growth hormones (Hartree *et al.* 1968).

Dixon & Li (1966) showed that human growth hormone retains its biological activity after reduction of the disulphide bonds and treatment with iodoacetamide, and Mills & Wilhelmi (1965) demonstrated that ox growth hormone retains its biological activity after cleavage of half the disulphide bonds. The differences in the sizes of disulphide rings and in the amino acid sequences around the half-cystine residues of human and horse growth hormones provide additional evidence that the biological activity is not dependent on the position of the disulphide bonds.

We are indebted to Dr R. N. Perham and Mr S. van Heyningen of this Department and to Dr A. G. Weeds and other members of the Medical Research Council Laboratory of Molecular Biology, Cambridge, for advice and helpful discussions. Miss M. B. Thomas prepared the growth hormone, and a grant in support of this work was provided by the Medical Research Council. We thank Professor F. G. Young, F.R.S., for his encouragement.

REFERENCES

- Ambler, R. P. (1963). *Biochem. J.* **89**, 349.
- Ambler, R. P. & Brown, L. H. (1967). *Biochem. J.* **104**, 786.
- Brown, J. R. & Hartley, B. S. (1963). *Biochem. J.* **89**, 59p.
- Brown, J. R. & Hartley, B. S. (1966). *Biochem. J.* **101**, 214.
- Dixon, J. S. & Li, C. H. (1966). *Science*, **154**, 785.
- Gray, W. R. (1967). In *Methods in Enzymology*, vol. 11, p. 469. Ed. by Hirs, C. H. W., Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Gray, W. R. & Hartley, B. S. (1963a). *Biochem. J.* **89**, 59p.
- Gray, W. R. & Hartley, B. S. (1963b). *Biochem. J.* **89**, 379.
- Hartree, A. S., Kovačić, N. & Thomas, M. (1965). *J. Endocrin.* **33**, 249.
- Hartree, A. S., Mills, J. B., Welch, R. A. S. & Thomas, M. (1968). *J. Reprod. Fertil.* (in the Press).
- Hirs, C. H. W. (1956). *J. biol. Chem.* **219**, 611.

- Li, C. H., Liu, W. K. & Dixon, J. S. (1966). *J. Amer. chem. Soc.* **88**, 2050.
- Michl, H. (1951). *Mh. Chem.* **82**, 489.
- Mills, J. B. (1967). *Nature, Lond.*, **213**, 631.
- Mills, J. B. & Wilhelmi, A. E. (1965). *Endocrinology*, **76**, 522.
- Offord, R. E. (1966). *Nature, Lond.*, **211**, 591.
- Raben, M. S. (1959). *Recent Progr. Hormone Res.* **15**, 71.
- Raftery, M. A. & Cole, R. D. (1963). *Biochem. biophys. Res. Commun.* **10**, 467.
- Ryle, A. P., Sanger, F., Smith, L. G. & Kitai, R. (1955). *Biochem. J.* **60**, 541.
- Santomé, J. A., Wolfenstein, C. E. M., Biscoglio, M. & Paladini, A. C. (1966). *Arch. Biochem. Biophys.* **116**, 19.
- Santomé, J. A., Wolfenstein, C. & Paladini, A. C. (1965a). *Biochem. biophys. Res. Commun.* **20**, 482.
- Santomé, J. A., Wolfenstein, C. E. M. & Paladini, A. C. (1965b). *Biochim. biophys. Acta*, **111**, 342.
- Saxena, B. B. & Henneman, P. H. (1966). *Endocrinology*, **78**, 561.
- Toennies, G. & Kolb, J. J. (1951). *Analyt. Chem.* **23**, 823.
- Waley, S. G. & Watson, J. (1953). *Biochem. J.* **55**, 328.
- Wallis, M. (1966). *Biochim. biophys. Acta*, **115**, 423.