BBA 65832

ON THE HOMOLOGY OF THE ACTIVE-SITE PEPTIDES OF LIVER CARBOXYLESTERASES

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(Received August 9th, 1968)

SUMMARY

1. Highly purified preparations of liver carboxylesterases (carboxylic ester hydrolases, EC 3.1.1.1) from pig, sheep, ox and chicken were stoichiometrically labelled with [^{32}P]DFP, and then subjected to peptic digestion.

2. Radioactive peptides were isolated from the peptic digests by chromatography on Sephadex G-25, paper chromatography and high voltage electrophoresis. For each species, an octapeptide was isolated as the major radioactive peptide.

3. Amino acid analyses of pig and sheep octapeptides were identical with the previously published analysis of the corresponding octapeptide from horse liver carboxylesterase. Analyses of ox and chicken octapeptides both indicated single amino acid substitutions when compared with the horse octapeptide.

4. The two amino acid substitutions were located by conventional sequencing procedures. In the ox octapeptide, alanine replaces the glycine three residues from the labelled serine towards the C-terminal. In the chicken peptide, isoleucine replaces the glutamic acid residue four removed from the serine towards the C-terminal. The possible significance of the amino acid substitutions is discussed in terms of other properties of the enzymes.

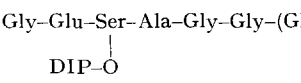
INTRODUCTION

Carboxylesterases (carboxylic ester hydrolases, EC 3.1.1.1) constitute one of several groups of enzymes which catalyse the hydrolysis of carboxylic esters and are widely distributed in mammalian tissues. Carboxylesterases are distinguished from other classes of esterase by substrate and inhibitor studies. One of the important properties of carboxylesterases is their inhibition by low concentrations of DFP. BOURSNELL AND WEBB[1] inhibited a partially purified horse liver carboxylesterase with [^{32}P]DFP, and found the incorporation of 1 atom of P/96 000 g of protein.

Many hydrolytic enzymes have been found to react stoichiometrically with DFP, yielding derivatives which are completely inactive and in which one serine

Abbreviation: DIP-, diisopropylphosphoryl-.

residue is phosphorylated[2]. The sequence of amino acids adjacent to this serine has been determined using [^{32}P]DFP. JANSZ, POSTHUMUS AND COHEN[3,4] labelled horse liver carboxylesterase with [^{32}P]DFP and digested the resulting diisopropylphosphoryl-enzyme (DIP-enzyme) with pepsin. They isolated an octapeptide from the digest as the major radioactive peptide. The amino acid sequence of this peptide, determined by dinitrophenylation, Edman degradation and partial acid hydrolysis was given as



The present species comparison of active site sequences of carboxylesterases was instigated for several reasons. For the animal proteinases investigated, the sequence Gly-Asp-Ser-Gly was found, while for the esterases, the sequence found was Glu-Ser-Ala (ref. 2), where the starred serine is the residue phosphorylated by DFP. The carboxylesterases are more efficient than the serine proteinases by several orders of magnitude in their ability to hydrolyse simple esters (J. K. STOOPS AND B. ZERNER, unpublished results). If the sequence Glu-Ser-Ala is ubiquitous in esterases, it may be responsible, at least in part, for this difference in reactivity between esterases and proteinases. A detailed species comparison of physical and catalytic properties of carboxylesterases is currently in progress in this laboratory. Differences in amino acid sequence close to the reactive serine residue might comment on differences observed in such properties.

A preliminary report of some of this work has been published[5]. Carboxylesterases were labelled with [^{32}P]DFP and subjected to partial hydrolysis by HCl or by pepsin. Patterns of the radioactive peptides produced were obtained by radioautography of ionograms and chromatograms of the digests. Electrophoresis of the partial acid hydrolysates at pH 3.6 gave identical patterns of five radioactive peptides for each of the five liver carboxylesterases examined (pig, horse, ox, sheep and chicken). This indicated that residues adjacent to the labelled serine were identical. Chromatography of the peptic digests revealed differences in amino acid sequences further removed from the active serine. Major radioactive peptides from pig, horse and sheep enzymes were identical, as judged by two-dimensional chromatography in *n*-butanol–acetic acid–water (4:1:5, by vol., upper phase), and *n*-butanol–pyridine–acetic acid–water (15:10:3:12, by vol.). The ox peptide appeared slightly different and the chicken peptides markedly different from those of the other species in terms of their chromatographic behaviour in *n*-butanol–acetic acid–water.

MATERIALS AND METHODS

Liver carboxylesterases from pig, horse, ox, sheep and chicken were purified from acetone or chloroform–acetone powders by procedures similar to those described for pig liver carboxylesterase (ref. 6)*. Starch and polyacrylamide gel electrophoresis demonstrated that the enzymes were in a high state of purity. The specific activity of the carboxylesterases was determined in a standard assay against ethyl butyrate[6].

* Liver esterases were prepared by the following workers: pig, J. K. STOOPS; horse, E. A. BENNETT; ox and sheep, M. T. C. RUNNEGAR, K. SCOTT; chicken, P. A. INKERMAN.

[^{32}P]DFP was obtained from the Radiochemical Centre, Amersham, Great Britain, as a solution in propylene glycol (0.56 mg/ml; 200 μC/ml). Twice crystallized pepsin was obtained from Worthington Biochemicals Corporation. Components of chromatography solvents and electrophoresis buffers were analytical grade reagents. Sephadex G-25 was obtained from Pharmacia, Uppsala, Sweden. Pyridine was redistilled before use. Constant boiling HCl was distilled 3 times before use. Dansyl chloride and phenyl isothiocyanate were obtained from British Drug Houses Ltd., Great Britain. Phenyl isothiocyanate was redistilled before use. A highly purified preparation of ficin was supplied by Mr. A. A. Kortt. Porcine carboxypeptidase A (thrice crystallized) was prepared by Dr. M. A. McDowall by a modification of the procedure of FOLK AND SCHIRMER[7].

Assay of enzyme activity

The activity of the carboxylesterases was determined spectrophotometrically before and after treatment with [^{32}P]DFP, using *p*-nitrophenyl acetate as substrate. Assays were performed in 0.1 M phosphate buffer (pH 7.0) at 25° on a Cary 14 recording spectrophotometer by following the release of *p*-nitrophenolate ion at 400 mμ. The ultraviolet spectra of the proteins were also determined on the Cary 14 spectrophotometer.

Preparation of DIP-enzymes

The enzymes were made up as solutions (6 mg/ml) in 0.05 M phosphate buffer (pH 7.0). [^{32}P]DFP was added in a 1.5-fold molar excess, assuming an equivalent weight of 80 000 for the carboxylesterases[6]. Reaction was allowed to continue at room temperature (approx. 20°) for 15 h with ox, pig and chicken enzymes, and 21 h with the sheep enzyme. The inhibited enzymes were dialysed against 5 changes each of 100 vol. of distilled water, and lyophilized.

Measurement of radioactivity

Radioactivity of labelled proteins and peptides was determined by liquid scintillation counting on a Nuclear Chicago Mark I Liquid Scintillation System, Model 6080. Routinely, 25 μl of aqueous solution was added to 14 ml of scintillation fluid, containing toluene, Triton nonionic detergent, Liquiflor scintillator and Nuclear Chicago solution (960:700:40:3.4, by vol.). Counting efficiency was estimated at 74%. Internal standards were prepared from the supplied [^{32}P]DFP solution, and used throughout the purification procedure. Radioactive peptides were located on paper ionograms and chromatograms by radioautography on Ilford X-Ray film.

Peptic digestion

Using previously determined conditions[5] (pH 2; 6 mg/ml DIP-enzyme; 0.3 mg/ml pepsin; 3 h; 37°), pig, sheep and ox enzymes gave predominantly one radioactive peptide. Pilot experiments indicated that more drastic conditions were required for the chicken enzyme (pH 2; 6 mg/ml DIP-enzyme; 1 mg/ml pepsin; 36 h; 37°). The digestion was terminated by the addition of pyridine, and the digests lyophilized.

Chromatography on Sephadex G-25

Sephadex G-25 chromatography of the peptic digests was performed on a

1.7 cm × 210 cm column, equilibrated with 0.8% ammonium bicarbonate (pH 8). The eluant was monitored by measuring the radioactivity and absorbance (220 mμ and 280 mμ) of the 6 ml fractions. The fractions containing radioactivity were pooled, counted and lyophilized.

Paper chromatography and high voltage electrophoresis

Both chromatography and electrophoresis were run on Whatman 3MM paper, samples being applied as streaks. Chromatography was carried out in two solvent systems, *n*-butanol–pyridine–acetic acid–water (15:10:3:12, by vol.) and *n*-butanol–acetic acid–water (40:6:15, by vol.). Electrophoresis was carried out in pyridine–acetate buffers at pH 3.6 (pyridine–acetic acid–water, 1:10:189, by vol.) and pH 5.6 (pyridine–acetic acid–water, 25:7:1968, by vol.), with a potential gradient of approx. 70 V/cm. Radioactive peptides were located by radioautography, and the strips were cut out and eluted with 10% pyridine. The eluates were counted and dried *in vacuo*. The remainder of the chromatogram or ionogram was then sprayed with ninhydrin as a guide to the purification achieved by the procedure, and as a purity check.

Amino acid analyses

Samples of purified peptides (20–50 nmoles) were hydrolysed with constant boiling HCl at 110° for 40 h unless otherwise specified. Amino acid analyses were performed on a Technicon Auto-Analyser modified for amino acid analysis by the procedure of MOORE AND STEIN[8].

Methods of sequence determination

N-Terminal amino acids were determined by the method of GRAY AND HARTLEY[9]. Dansylamino acids were separated by thin-layer chromatography[10] and by electro-

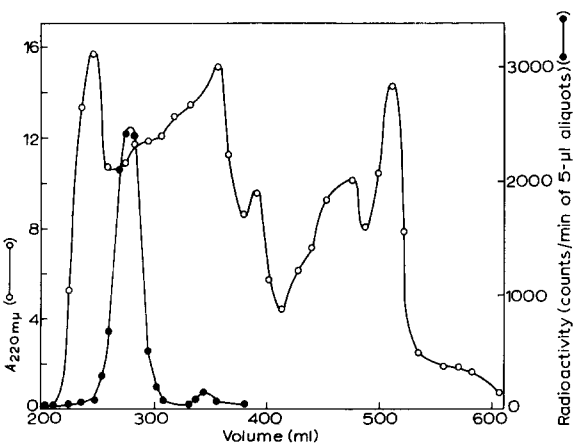


Fig. 1. Chromatography of the peptic digest of [^{32}P]DIP-pig liver carboxylesterase on Sephadex G-25. The peptic digest of 250 mg of DIP-pig liver carboxylesterase was applied in 2.6 ml to a 1.7 cm × 210 cm column, equilibrated and eluted with 0.8% ammonium bicarbonate (pH 8). Flow rate 20 ml/h; 6-ml fractions. Pepsin was eluted at about 240 ml. Fractions between 256 ml and 304 ml were pooled. ○—○, absorbance at 220 mμ; ●—●, radioactivity in counts/min of 5-μl aliquots.

phoresis at pH 4.4 when necessary to distinguish dansylserine from dansylglutamic acid. Dansylamino acids were identified by comparison with appropriate standards. Sequential degradation of the peptides was performed by the Edman method as modified by MARGOLIASH[11]. Both trifluoroacetic acid and glacial acetic acid–concentrated HCl (5:1, by vol.) were used as cyclizing agents. Carboxypeptidase A digestions were carried out at 40° in 0.25 M ammonium bicarbonate (pH 7.8), with a peptide concentration of 1 mM. Amino acids released were estimated on the amino acid analyser.

RESULTS

Stoichiometry of the reaction with DFP

The absorbance at 280 mμ and the phosphorus content of the DIP-enzymes were determined after exhaustive dialysis (Table I). The equivalent weights of the enzymes in the reaction with DFP have been calculated for those enzymes where the correlation between absorbance at 280 mμ and protein concentration in mg/ml has been measured. The percentage of the original activity remaining after the DFP treatment is also included in Table I.

TABLE I

STOICHIOMETRY OF THE REACTION OF LIVER CARBOXYLESTERASES WITH [^{32}P]DFP

Species	*(P)/$A_{280\ m\mu}$ of DIP-enzymes (μM/absorbance unit)*	*Equivalent weight (g protein/equiv. DFP)*	*% of initial activity*
Horse	11.6 (ref. 5)		
Pig	9.7	79 000	0.04
Ox	10.5	70 000	0.03
Sheep	9.6		<0.1
Chicken	9.9	82 000	0.16

Column chromatography of peptic digests

Fig. 1 shows a typical Sephadex G-25 elution profile. In all cases, most of the radioactivity was eluted from the column in a single peak between 250 and 300 ml. Table II gives details of the amounts of liver carboxylesterases used, and the amounts of radioactivity recovered in the pooled peaks from the Sephadex column.

Paper electrophoresis and chromatography

Radioactive peptides were then subjected to electrophoresis at pH 5.6. Pig, sheep and ox peptide ionograms showed one major band, but the chicken peptide ionogram revealed several radioactive peptides. Further purification was then achieved by chromatography in *n*-butanol–pyridine–acetic acid–water (Table III). Purity checks by electrophoresis at pH 3.6 and *n*-butanol–acetic acid–water chromatography indicated that P3a, S2a and O2a were almost homogeneous by these criteria.

For P3a and S2a, preparative scale *n*-butanol–acetic acid–water chromato-

TABLE II

YIELDS OF RADIOACTIVE PEPTIDES AFTER CHROMATOGRAPHY ON SEPHADEX G-25

Species	*Amount enzyme used (mg)*	*Amount [^{32}P]DIP-peptide in pooled peak (μmoles)*
Pig	250	2.8
Sheep	200	2.3
Ox	160	2.3
Chicken	500	6.0

graphy resulted in a slight improvement in the amino acid analysis. Based on the above criteria, the chicken peptides were still heavily contaminated, and three further purification steps proved to be necessary: electrophoresis at pH 3.6, chromatography in *n*-butanol–acetic acid–water and re-electrophoresis at pH 5.6. Two chicken peptides, C3b1a1 (0.4 μmole), and C4a1a1 (0.3 μmole), were thus isolated in a satisfactory state of purity. Several of the minor peptides were purified in a similar manner.

The difficulty experienced in purification of the chicken peptides was presumably due to the drastic conditions of the peptic hydrolysis. Considerable losses were sustained in every purification step because of the loss of [^{32}P]diisopropylphosphate. Diisopropylphosphate is readily lost from labelled peptides, presumably because of intramolecular catalysis by the adjacent amide group[12]. Diisopropylphosphate has a high electrophoretic mobility at pH 3.6 and 5.6, and a high R_F in both *n*-butanol–acetic acid–water and *n*-butanol–pyridine–acetic acid–water. Radioactivity corresponding to diisopropylphosphate was evident in all radioautograms. The 10% pyridine used in the elution of peptides from the paper may have catalysed the loss. It was found that treatment of the DIP-peptides with 0.02 M NaOH at 40° for 3 h was sufficient for complete removal of the diisopropylphosphoryl group.

Amino acid analyses of radioactive peptides

The amino acid analyses and N-terminal residues of the major peptides are given in Table IV. From these results, it is clear that P3a and S2a have the same

TABLE III

PURIFICATION OF RADIOACTIVE PEPTIDES

Species	*Major peptides after electrophoresis at pH 5.6*	*Amount (μmoles)*	*Peptides after n-butanol–pyridine–acetic acid–water chromatography*	*Amount (μmoles)*
Pig	P3	1.7	P3a	1.38
Sheep	S2	1.4	S2a	1.05
Ox	O2	1.6	O2a	0.82
			O2b	0.39
Chicken	C3	1.5	C3a	0.5
			C3b	0.7
	C4	0.9	C4a	0.8

TABLE IV

AMINO ACID COMPOSITION AND N-TERMINAL RESIDUES OF MAJOR RADIOACTIVE PEPTIDES

Peptide	*Amino acid composition*					*N-Terminal residue*
	Gly	*Glu*	*Ser*	*Ala*	*Ile*	
P3a	2.87	2.02	1.03	1.00	(0.05)	Gly
S2a	2.87	1.92	1.03	1.00	(0.10)	Gly
O2a	2.00	2.03	1.68	2.00	(0.03)	Gly
O2bb*	1.89	1.81	0.68	2.19	(<0.1)	Gly
C3b1a1**	2.98	1.14	1.66	1.10	0.96	Gly
C4a1a1**	3.00	1.92	0.96	1.00	(0.10)	Gly

* Major peptide after *n*-butanol–acetic acid–water chromatography of O2b.
** Hydrolysis for 70 h.

amino acid composition as the DIP-peptide from the peptic digest of horse liver carboxylesterase[3], *i.e.* Gly_3, Glu_2, Ser_2 and Ala_1. O2a has the composition Gly_2, Glu_2, Ser_2, Ala_2, probably indicating a conservative substitution of alanine for glycine. C3b1a1 has the composition Gly_3, Glu_1, Ser_2, Ala_1, Ile_1, signifying a non-conservative substitution of isoleucine for glutamic acid.

Amino acid sequences

Edman degradation of the major peptides from each of the four species gave similar results. Table V shows results obtained with S2a. The N-terminal sequence of the four octapeptides was thus found to be Gly–Glu–Ser. All attempts to remove the third residue were unsuccessful (Table V). Similar difficulties were experienced by JANSZ, POSTHUMUS AND COHEN[4], and by OOSTERBAAN *et al.*[13]. Treatment of the octapeptides with NaOH to remove the diisopropylphosphoryl group yielded a product which would not react with phenyl isothiocyanate.

Table VI shows the results obtained by digestion of the radioactive peptides with carboxypeptidase A. For P3a, S2a and O2a, serine alone could be removed, even under drastic conditions. However, other work in this laboratory has shown that porcine carboxypeptidase A cleaves C-terminal glutamic acid only very slowly. Results obtained with the chicken peptides are much more informative, and allow identificatio nof the C-terminals equence of the octapeptide C3b1a1 as Ala–Gly–Gly–Ile-Ser.

Research on the specificity of the proteolytic enzyme ficin being conducted in this laboratory indicated that it might be suitable for cleavage of the ox peptide O2a

TABLE V

EDMAN DEGRADATION OF MAJOR SHEEP PEPTIDE

Edman step number	*N-Terminal residue*	*Amino acid composition*
0	Gly	Gly (2.87), Glu (1.92), Ser (1.63), Ala (1.00)
2	Ser	*Gly* (2.18), *Glu* (1.15), Ser (1.53), Ala (1.00)
4	Ser	Gly (2.20), Glu (1.06), Ser (1.27), Ala (0.84)

TABLE VI

DETERMINATION OF CARBOXYL TERMINAL SEQUENCES BY DIGESTION WITH CARBOXYPEPTIDASE A

Peptide	*Conditions of digestion*	*Amino acids released (residues)*
P3a	26 h; 50 μM CPA*	Ser (0.8)
S2a	28 h; 50 μM CPA	Ser (0.84)
O2a	12 h; 20 μM CPA	Ser (0.65)
C3b1a1	45 min; 20 μM CPA	Ser (0.68), Ile (0.14)
	90 min; 20 μM CPA	Ser (1.0), Ile (0.68)
C4a1a1	150 min; 20 μM CPA	Gly (0.44), Ala (0.10)
	12.5 h; 40 μM CPA	Gly (1.71), Ala (0.45)

* CPA, carboxypeptidase A.

into smaller fragments. O2a (1 mM; 0.13 μmole) was digested with ficin (approx. 10 μM) at 30° for 46 h in 0.1 M phosphate buffer (pH 6.0) containing 0.1 M mercaptoethanol and 1 mM EDTA. Electrophoresis of the digest at pH 5.6 and radioautography revealed the disappearance of the spot corresponding to O2a and the appearance of a new radioactive spot with a decreased negative charge. This new spot had glycine as the N-terminal residue. Two radioactive peptides were isolated from the digest by *n*-butanol–acetic acid–water chromatography and subjected to amino acid analysis. The following results were obtained:

O2aF3 Gly (2.1), Glu (1.0), Ser (0.3), Ala (1.0),
O2aF4 Gly (2.0), Glu (0.9), Ser (0.9), Ala (2.2).

Apart from the somewhat anomalous results for serine, the peptides differ by one alanine residue. Carboxypeptidase A digestion of peptides O2a F3 and O2a F4 released glycine and alanine, respectively. Together with the amino acid analyses of O2a, O2bb, O2a F3 and O2a F4, these results establish the C-terminal sequence of O2a as–Gly–Ala–Glu–Ser.

Table VII summarizes results obtained from sequence studies of the four species and includes the sequence determined for the horse octapeptide[4].

TABLE VII

AMINO ACID SEQUENCES OF ACTIVE-SITE PEPTIDES

Species	*Amino acid sequence*
Horse (ref. 4)	Gly–Glu–Ser–Ala–Gly–Gly–(Glu, Ser)
Pig, sheep	Gly–Glu–Ser–(Ala, Gly, Gly, Glu)–Ser
Ox	Gly–Glu–Ser–Ala–Gly–Ala–Glu–Ser
Chicken	Gly–Glu–Ser–Ala–Gly–Gly–Ile–Ser

DISCUSSION

From the sequence information given in Table VII, and from a consideration of the peptide patterns obtained by partial acid hydrolysis of the DIP-enzymes[5], the following conclusions can be drawn:

(1) Horse, pig and sheep liver carboxylesterases have identical sequences around the active serine residue, *viz.* Gly-Glu–Sêr–Ala–Gly-Gly-Glu-Ser.

(2) Ox liver carboxylesterase has a substitution of alanine for glycine three residues from the reactive serine.

(3) Chicken liver carboxylesterase has a substitution of isoleucine for glutamic acid four residues from the reactive serine.

The conservative substitution in the ox enzyme is quite common and involves the change of only one nucleotide in the coding triplet: GG(A, G, C, U) codes for glycine, GC(A, G, C, U) for alanine. The non-conservative substitution in the chicken enzyme involves the change of more than one nucleotide in the coding triplet for glutamic acid (glutamic acid, GA(A, G); isoleucine, AU(A, C, U)). Examination of the sequences of 19 cytochromes *c* reveals two examples in which isoleucine and glutamic acid can occupy the same allelic position[14]. The more radical difference in sequence observed with the chicken peptide is not unreasonable on evolutionary grounds.

The sequence Glu-Sêr-Ala at the active site is present in all five liver carboxylesterases, and may be important in the mechanism of hydrolysis of esters. Although the purified preparations exist in multiple forms, only one peptide was isolated containing the active serine residue. Therefore, the multiple forms all appear to have the same sequence about this serine.

Similarities in active site sequences (Table VII) and in stoichiometry of reaction with DFP (Table I) are paralleled by other similarities. All of the liver carboxylesterases react with *p*-nitrophenyl dimethylcarbamate to give a "burst" of *p*-nitrophenol and a relatively stable dimethylcarbamyl-enzyme. Amino acid compositions are similar. Detailed kinetic and specificity studies on pig[15] and ox[16] liver carboxylesterases have been made, permitting a comment on the possible significance of the substitution of alanine for glycine as a specificity determinant. Pig liver carboxylesterase reacts with *o*- and *p*-nitrophenyl dimethylcarbamates at about the same rate. However, the *ortho*-substituted ester reacts more rapidly than the *para*- with the ox enzyme by a factor of 10. Similarly, k_{cat} values for the pig liver carboxylesterase-catalysed hydrolysis of *o*- and *p*-nitrophenyl butyrates are identical. With ox liver carboxylesterase, k_{cat} for the *ortho*-ester is twice that for the *para*-. The factor of two in k_{cat} values probably represents a much larger factor in the acylation reaction. Therefore, a *para*-nitro group on the phenyl ring would appear to cause considerable steric hindrance to acylation of the ox enzyme but not to acylation of the pig enzyme. The specificity of the sheep enzyme resembles that of the pig enzyme[16]. It seems likely that the methyl side chain of the alanine present in the ox but absent in the pig and sheep is the source of the observed steric hindrance.

Chicken liver carboxylesterase has a molecular weight of about 80 000, compared to about 160 000 for the other species (P. A. INKERMAN AND B. ZERNER, unpublished results). Pig and ox enzymes exist as dimers at neutral pH and reasonably high concentrations, but as monomers (mol. wt. about 80 000) at low pH and low concentrations[16–18]. Dissociation of dimers at low pH indicates that an ionized carboxyl group might be important in maintaining the dimeric structure. The substitution of isoleucine for glutamic acid in the chicken may then explain why the chicken enzyme does not form dimers.

ACKNOWLEDGEMENTS

We acknowledge with thanks the indefinite loan of a Cary 14 spectrophotometer from the Wellcome Trust, London. This work was supported in part by the A.R.G.C. (Australia) and in part by grant GM 13759 from the Institute of General Medical Sciences of the National Institutes of Health (U.S.A.). Two of us (R. C. A. and J. DE J.) acknowledge a Commonwealth Postgraduate Award and a CSIRO Postgraduate Studentship respectively.

REFERENCES

1 J. C. BOURSNELL AND E. C. WEBB, *Nature*, 164 (1949) 875.
2 M. L. BENDER AND F. J. KÉZDY, *Ann. Rev. Biochem.*, 34 (1965) 49.
3 H. S. JANSZ, C. H. POSTHUMUS AND J. A. COHEN, *Biochim. Biophys. Acta*, 33 (1959) 387.
4 H. S. JANSZ, C. H. POSTHUMUS AND J. A. COHEN, *Biochim. Biophys. Acta*, 33 (1959) 396.
5 R. L. BLAKELEY, J. DE JERSEY, E. C. WEBB AND B. ZERNER, *Biochim. Biophys. Acta*, 139 (1967) 208.
6 D. J. HORGAN, E. C. WEBB AND B. ZERNER, *Biochem. Biophys. Res. Commun.*, 23 (1966) 18.
7 J. E. FOLK AND E. W. SCHIRMER, *J. Biol. Chem.*, 238 (1963) 3884.
8 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 211 (1954) 893.
9 W. R. GRAY AND B. S. HARTLEY, *Biochem. J.*, 89 (1963) 379.
10 D. MORSE AND B. L. HORECKER, *Anal. Biochem.*, 14 (1966) 429.
11 E. MARGOLIASH, *J. Biol. Chem.*, 237 (1962) 2161.
12 G. L. SCHMIR AND C. ZIOUDROU, *Biochemistry*, 2 (1963) 1305.
13 R. A. OOSTERBAAN, P. KUNST, J. VAN ROTTERDAM AND J. A. COHEN, *Biochim. Biophys. Acta*, 27 (1958) 556.
14 R. V. ECK AND M. O. DAYHOFF, *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, Maryland, 1966, p. 171.
15 D. J. HORGAN, Ph. D. Thesis, University of Queensland, Brisbane, 1966.
16 M. T. C. RUNNEGAR, Ph. D. Thesis, University of Queensland, Brisbane, 1968.
17 D. BARKER AND W. P. JENCKS, *Federation Proc.*, 26 (1967) 452.
18 H. C. BENÖHR AND K. KRISCH, *Z. Physiol. Chem.*, 348 (1967) 1115.