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# STUDIES ON CRYSTALLINE LACTATE DEHYDROGENASE FROM CARDIAC AND SKELETAL MUSCLE OF PLAICE (*PLEURONECTES PLATESSA*) WITH PARTICULAR REFERENCE TO TEMPERATURE

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SUMMARY

1. The isolation of the single lactate dehydrogenase (L-lactate:$NAD^+$ oxidoreductase, EC 1.1.1.27) from both the cardiac and skeletal muscle of plaice is described.

2. These enzymes appear to be homogeneous and identical to one another, as judged by ultracentrifugal analysis and starch-gel electrophoresis. The two enzymes are indistinguishable on the basis of their reactivity with coenzyme analogues, and they are similar in amino acid composition.

3. There is marked substrate (pyruvate) inhibition of the plaice cardiac/skeletal muscle lactate dehydrogenase measured at 10°, and this is similar in extent to the pyruvate inhibition of beef H4 lactate dehydrogenase (LDH-1) measured at 35°. Beef H4 lactate dehydrogenase acting at 10° exhibits a greater degree of pyruvate inhibition than does plaice muscle lactate dehydrogenase at this temperature. Beef M4 lactate dehydrogenase (LDH-5) shows little pyruvate inhibition at 35°.

4. The marked product (L-lactate) inhibition of plaice muscle lactate dehydrogenase at 10° is compared with that of beef H4 lactate dehydrogenase.

5. The findings on substrate and product inhibition of plaice muscle lactate dehydrogenase at low temperature are discussed in the context of lactate dehydrogenase isoenzyme function.

## INTRODUCTION

It has been known for some time that pyruvate (substrate) inhibition of isoenzymes of lactate dehydrogenase (L-lactate:$NAD^+$ oxidoreductase, EC 1.1.1.27) is affected by temperature[1]. At low temperatures (6°), mammalian lactate dehydrogenase isoenzymes show greater pyruvate inhibition than at high temperatures (35–40°), and the marked differences in pyruvate inhibition between LDH-1 and LDH-5 which exist at 25° become appreciably less at 35–40° (*i.e.* at physiological temperatures for mammals and birds)[2]. These findings have led to some criticism of the view that the

functional significance of different lactate dehydrogenase isoenzymes lies in the differing degrees of substrate inhibition which they exhibit[3].

It has also been shown recently that LDH-1 exhibits much greater product (L-lactate) inhibition than does LDH-5 at 25°, and it has been considered that this inhibitory effect may be significant in evaluating the functional roles of lactate dehydrogenase isoenzymes[4]. There is however conflicting evidence as to the extent of differences in product inhibition at 37° (refs. 3, 5).

Many flatfish appear to have only a single lactate dehydrogenase isoenzyme common to both cardiac and skeletal muscle[6], and the object of the present work was first to isolate and study the properties of lactate dehydrogenase isolated from cardiac and skeletal muscle of plaice and then to compare substrate and product inhibition of plaice muscle lactate dehydrogenase at low temperatures with that of mammalian lactate dehydrogenases.

## MATERIALS AND METHODS

Plaice (*Pleuronectes platessa*) were obtained from local inshore fishermen and were maintained in the unit aquarium at 10° for at least 1 week prior to use. For the preparation of cardiac muscle lactate dehydrogenase, large quantities of plaice "heads" (which contained the heart) were obtained from John Bruce, Fish Merchants, Aberdeen, within 12 h of the death of the fish. These fish had been kept on ice from the time of capture until filleting and decapitation. Hearts were collected daily for 2 weeks, each daily supply being taken through the initial stages of extraction (to 25–65% $(NH_4)_2SO_4$ suspension) at once. When about 1 kg of hearts had been worked up to this stage, the suspensions were combined and the lactate dehydrogenase preparation completed.

Chemicals were obtained from the following sources: NADH disodium salt, $NAD^+$, nicotinamide–hypoxanthine dinucleotide, reduced nicotinamide–hypoxanthine dinucleotide, 3-pyridine aldehyde–adenine dinucleotide, thionicotinamide–adenine dinucleotide, 3-acetylpyridine–hypoxanthine dinucleotide, sodium pyruvate (dimer free), Tris, and lactate dehydrogenase (Type II from rabbit muscle) from Sigma Chemical Co. (London). Beef H4 (Bande 1) and beef M4 (Bande 5) lactate dehydrogenases were from the Boehringer Corp. (London) and DEAE-cellulose (DE32 microgranular) from H. Reeve Angel and Co. $(NH_4)_2SO_4$ "specially low in heavy metals for enzyme work" and other chemicals of A.R. grade were from British Drug Houses (Poole, Dorset).

### *Measurement of lactate dehydrogenase activity*

This was done as described previously[7]. For measurements at subambient temperature, water was cooled with a Townson–Mercer laboratory refrigeration unit and was circulated through the cell housing with a circon (Baird and Tatlock). Misting (condensation) of the cells was prevented by a steady flow of dry gas (nitrogen) through the cell compartment (about 100 ml/min).

### *Determination of protein content*

During the purification of the enzyme, this was carried out spectrophotometrically assuming a specific extinction coefficient ($E_{1\,cm}^{1\%}$) at 276 mμ of 10. For crystalline lactate dehydrogenase, a specific extinction coefficient of 13.5 was used[8].

*Product inhibition of plaice muscle lactate dehydrogenase at 10°*

Product inhibition by L-lactate was examined at a series of lactate concentrations up to 80 mM using pyruvate concentrations up to 0.1 mM. The reaction mixtures were buffered at pH 7.4 in 0.1 M phosphate buffer, and the NADH concentration was 0.15 mM.

*Starch-gel electrophoresis*

This was carried out at pH 8.8 as described by POULIK[9]. The gels were stained for protein with 0.1% nigrosine.

*Amino acid analysis of plaice heart and skeletal muscle lactate dehydrogenase*

2.0 ml of the crystalline suspension of each enzyme (containing about 30 mg protein) were centrifuged at 20 000 × *g* for 30 min. The protein was dissolved in water and dialysed against water overnight. The nondiffusable material was freeze dried and was either hydrolysed directly or subjected to performic acid oxidation, essentially according to the procedure of HIRS[10], and was then hydrolysed. Acid hydrolysis was carried out in twice glass-distilled 5.7 M HCl at 110 ± 1° for 24-, 48- and 72-h periods in evacuated sealed tubes which had been degassed as described by MOORE AND STEIN[11]. After hydrolysis the HCl was removed by rotary evaporation at 40°. The hydrolysate was then dissolved in 0.2 M citrate buffer (pH 2.2), containing 0.1 μmole norleucine per ml, and was examined on the Technicon amino acid analyser with a buffer system described by THOMAS[12].

*Tryptophan determination*

Tryptophan was determined by the method of SPIES AND CHAMBERS[13].

*Preparation of lactate dehydrogenase from plaice muscle and plaice heart*

The isolation procedure was based on the methods employed by PESCE *et al.*[8]. All operations were carried out at 5° except where otherwise stated. Plaice muscle (3.5 kg) was finely minced in a domestic mincer and stirred for 2 h with 6 l of 0.005 M Tris–HCl (pH 7.5). The mixture was strained through a double layer of muslin and was centrifuged at 5000 × *g* for 30 min. The supernatant was brought slowly to 70% satn. by the addition of solid $(NH_4)_2SO_4$, the pH of the mixture being maintained at 7.5 by the occasional addition of dilute $NH_3$. After standing for 2 h, the suspension was filtered overnight on large fluted papers.

The precipitate was scraped from the filter papers, dissolved in 0.005 M Tris–HCl (pH 7.5) and dialysed against the same buffer. Solid $(NH_4)_2SO_4$ was added until the solution reached 25% satn., and the suspension was stirred for 1 h. The suspension was centrifuged at 20 000 × *g* for 30 min, and the precipitate was discarded. Solid $(NH_4)_2SO_4$ was added to the supernatant solution until a level of 65% satn. was reached. The suspension was again stirred for 1 h and centrifuged at 20 000 × *g* for 30 min. The precipitate was dissolved in 0.005 M Tris–HCl (pH 7.5) and dialysed overnight against the same buffer.

The dialysed solution was brought to an acetone–water ratio of 1:3 (v/v) by the slow addition of −15° acetone with constant stirring. After standing at 0° for 15 min, the suspension was centrifuged for 15 min at 20 000 × *g*, and the inactive precipitate was discarded. Acetone (−15°) was then added slowly to the supernatant

with constant stirring until a final acetone–water ratio of 1:1 (v/v) was reached. After standing at 0° for 15 min, the suspension was again centrifuged at 20 000 × *g* for 15 min. The inactive supernatant was discarded, and the precipitate was extracted 3 times with 70 ml of 0.005 M Tris–HCl (pH 7.5). Any insoluble material in the combined extracts was removed by centrifuging at 20 000 × *g* for 15 min. Throughout the acetone fractionation the temperature was not allowed to rise above 0°.

The active material from the acetone fractionation was then chromatographed on DEAE-cellulose. The preparation and equilibration of the cellulose and the gradient used for developing the column were as described by PESCE *et al.*[14]. Three protein peaks were obtained from this column, all the lactate dehydrogenase activity being present in the final protein peak (salt concentration about 0.05 M). The active fractions were combined, brought to 70% satn. (pH 7.6) by the addition of solid $(NH_4)_2SO_4$ and allowed to stand overnight.

The precipitated lactate dehydrogenase was collected by centrifuging at 20 000 × *g* for 30 min and was dissolved in 20 ml of 0.005 M Tris–HCl. Solid $(NH_4)_2SO_4$ was added to the solution until a faint turbidity appeared (about 30% satn.); the solution was then allowed to stand at 5°. The enzyme began to crystallise within 1 h and crystallisation was allowed to proceed for several days, when the enzyme was harvested and stored as a suspension in $(NH_4)_2SO_4$ (40% satd.).

## RESULTS

### *Isolation and chemical properties of lactate dehydrogenase from skeletal and cardiac muscle*

The fractionation procedure used for skeletal muscle lactate dehydrogenase is shown in Table I. 280 mg of enzyme were obtained from 3.5 kg of muscle. A similar procedure was used in preparing the heart enzyme, 80 mg of enzyme being obtained from 1100 g of cardiac muscle. The acetone fractionation procedure can be omitted during the preparation of the skeletal muscle enzyme, but, as a comparison of the properties of the enzyme obtained from both tissues was intended, it was felt desirable to treat both tissues in the same way. The specific activity of the crystalline skeletal muscle enzyme was 310 and that of the cardiac muscle enzyme 315 μmoles NADH

TABLE I

EXTRACTION AND PURIFICATION OF PLAICE MUSCLE LACTATE DEHYDROGENASE

| | *Vol. (ml)* | *Total protein (g)* | *Specific activity (μmoles NADH oxidized per mg protein per min)* | *Total activity* |
|---|---|---|---|---|
| Initial extract | 5870 | 52.5 | 24 | $12 \cdot 10^5$ |
| 70% precipitate | 730 | 27 | 29 | $7.5 \cdot 10^5$ |
| 25–65% precipitate | 405 | 17.8 | 35 | $6.2 \cdot 10^5$ |
| 25–50% (v/v) acetone (−15°) | 230 | 6.7 | 54 | $3.6 \cdot 10^5$ |
| DEAE-cellulose fraction | 29 | 0.4 | 250 | $9.7 \cdot 10^4$ |
| Crystals | 20 | 0.28 | 300 | $8.4 \cdot 10^4$ |

oxidised per mg protein per min at 25°. Both enzyme preparations migrated as a single band of the same mobility on starch gel electrophoresis (pH 8.8), and the bands obtained by protein staining and by visualisation of lactate dehydrogenase activity were coincident. Moreover solutions of both enzymes gave a single symmetrical peak in the ultracentrifuge with an $s_{20,w}$ of 7.02.

The amino acid composition of the cardiac and skeletal muscle enzymes is shown in Table II, which summarises the results of hydrolyses carried out for 24, 48 and 72 h. The results have been corrected for hydrolytic losses of threonine and serine (5 and 10%, respectively) and for the slow rate of hydrolysis of certain bonds. For example, a valine–isoleucine bond may be presumed to occur in both enzymes because the yield of these amino acid residues increased by 10% between the 24- and 48-h hydrolysis times. The resistance to hydrolysis of this peptide bond is well known. The results for half cystine and methionine were obtained from the values for cysteic acid and methionine sulphone present in the hydrolysate of protein which had been previously oxidised with performic acid. The results given in Table II would indicate that there are no gross differences in amino acid composition between the two enzymes,although this does not necessarily imply structural identity.

*Reactivity with coenzyme analogues and $K_m$ values*

Evidence bearing on the identity of enzymes from cardiac and skeletal muscle of plaice has been obtained by examining their reactivity with coenzyme analogues. Different isoenzymes can be distinguished on the basis of their reactivity with coenzyme

TABLE II

AMINO ACID COMPOSITION OF PLAICE CARDIAC AND SKELETAL MUSCLE LACTATE DEHYDROGENASE

Values are expressed in residues per molecule assuming a molecular weight of 140 000. All values corrected.

| *Amino acid* | *Skeletal muscle lactate dehydrogenase* | *Cardiac muscle lactate dehydrogenase* |
|---|---|---|
| Asp | 123 | 120 |
| Thr | 59 | 58 |
| Ser | 93 | 91 |
| Glu | 128 | 125 |
| Pro | 49 | 47 |
| Gly | 108 | 104 |
| Ala | 64 | 66 |
| Val | 137 | 133 |
| Cyṡ | 20 | 20 |
| Met | 51 | 49 |
| Ile | 48 | 50 |
| Leu | 121 | 125 |
| Tyr | 19 | 20 |
| Phe | 28 | 29 |
| Lys | 108 | 110 |
| His | 51 | 53 |
| Arg | 44 | 42 |
| Trp | 22 | 23 |
| Total residues | 1273 | 1265 |

TABLE III

RELATIVE ACTIVITY WITH DIFFERENT COENZYME ANALOGUES OF CARDIAC AND SKELETAL MUSCLE LACTATE DEHYDROGENASE FROM PLAICE

All measurements made at 25°; coenzyme and coenzyme analogue concn. 0.2 mM; 0.1 M phosphate buffer (pH 7.4); wavelengths used: nicotinamide–hypoxanthine dinucleotide ($NHD^+$), 340 mμ; 3-pyridine aldehyde–adenine dinucleotide ($PyrAD^+$), 360 mμ; $NAD^+$, 340 mμ; 3-acetyl pyridine–adenine dinucleotide ($APAD^+$), 360 mμ; thionicotinamide–adenine dinucleotide ($TNAD^+$), 395 mμ. The subscripts H and L refer to the concentrations of pyruvate or lactate used H (high concn., equivalent to 0.1 M lactate and 3 mM pyruvate) and L (low concn., equivalent to 13 mM lactate and 0.3 mM pyruvate).

| *Dinucleotides* | *Skeletal* | *Cardiac* | *Substrate* |
|---|---|---|---|
| $NHD_H^+/NHD_L^+$ | 3.4 | 3.6 | Lactate |
| $PyrAD_L^+/NAD_L^+$ | 1.3 | 1.3 | Lactate |
| $NHDH_L/NADH_H$ | 0.21 | 0.19 | Pyruvate |
| $NADH_H/NADH_L$ | 1.6 | 1.8 | Pyruvate |
| $NHDH_H/NADH_H$ | 0.7 | 0.6 | Pyruvate |
| $APAD_L^+/TNAD_L^+$ | 27.0 | 26.0 | Lactate |

analogues[15]. The ratio of activities of plaice cardiac and skeletal muscle lactate dehydrogenases with some coenzyme analogues is shown in Table III. The values obtained with both enzymes are sensibly the same. This finding lends further support to the view that plaice heart and skeletal muscle lactate dehydrogenases are identical.

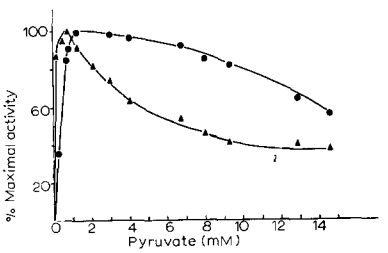
The two enzymes also gave similar values for $K_m$ (pyruvate) at different temperatures. These values were, at 10°, 0.1 and 0.11 mM and at 35°, 0.9 and 1 mM for skeletal and cardiac muscle lactate dehydrogenases, respectively.

*Heat and urea inactivation*

The thermostability of the cardiac and skeletal muscle isoenzymes and their stability to increasing concentrations of urea have been reported previously[7]. The isoenzymes are indistinguishable by both these criteria. It is possible to distinguish between avian and mammalian heart ($H_4$) and muscle ($M_4$) lactate dehydrogenases on the basis of their temperature stability[16] because the muscle enzymes are more thermolabile than are the heart enzymes. The cardiac/skeletal muscle lactate dehydrogenases from plaice are very thermolabile (inactivated in about 4 min at 56°) and in this respect resemble muscle type lactate dehydrogenases from birds and mammals.

*Substrate inhibition and product inhibition at different temperatures*

The influence of temperature on pyruvate inhibition of beef muscle (LDH-5) enzyme is shown in Fig. 1. At 10° the optimal pyruvate concentration (0.6 mM) is lower than at 35° (approx. 2 mM) and the lactate dehydrogenase activity is markedly inhibited at the lower temperature. This effect of temperature on pyruvate inhibition holds true for other lactate dehydrogenases that we have examined (plaice muscle lactate dehydrogenases shows little pyruvate inhibition at 25° or 35° *cf.* $NADH_H/NADH_L$ ratio (Table II), but is markedly inhibited at 10° (Fig. 2); we have observed similar results with a rabbit muscle lactate dehydrogenase preparation). In Fig. 2 pyruvate inhibition of plaice muscle lactate dehydrogenase at 10° (a relevant physiological temperature) is compared with pyruvate inhibition of beef muscle (LDH-5) and beef heart (LDH-1) at 35°. The substrate inhibition curves of beef heart lactate



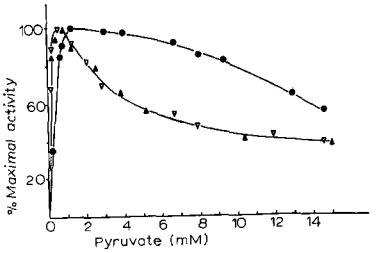
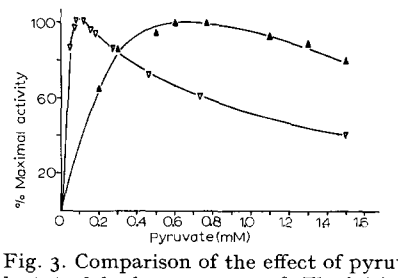




Fig. 1. Effect of pyruvate concentration on beef M4 lactate dehydrogenase at 10° and at 35°. The initial reaction velocities are plotted as percent of the maximal rate against the concentration of pyruvate at 10° (▲) and at 35° (●). NADH concn., 0.15 mM; 0.1 M phosphate buffer (pH 7.4).

Fig. 2. Comparison of the effect of pyruvate concentration on plaice muscle lactate dehydrogenase at 10° and on beef M4 and beef H4 lactate dehydrogenases at 35°. The initial reaction velocities are plotted as percent of the maximal rate against the concentration of pyruvate. ▲, plaice muscle; ●, beef M4; ▽, beef H4. NADH concn., 0.15 mM; 0.1 M phosphate buffer (pH 7.4).

dehydrogenase at 35° and of plaice muscle lactate dehydrogenase at 10° are similar with an optimal pyruvate concentration of about 0.6 mM and quite distinct from beef muscle at 35°. For comparative purposes the substrate inhibition curves at 10° of beef H4 and of plaice muscle lactate dehydrogenase are shown in Fig. 3, the substrate inhibition of the beef H4 is much more marked than that of the plaice enzyme; at this temperature beef H4 has a $K_m$ value of 49 $\mu$M which compares with a value of 100 $\mu$M for the plaice enzyme.

The effect of product (L-lactate) inhibition on the reduction of pyruvate by plaice muscle lactate dehydrogenase at 10° is shown in Fig. 4. Lineweaver–Burke plots of the data indicated that the inhibition was noncompetitive in nature; and Dixon plots of the data gave a $K_i$ value of 39 mM, this compares with reported $K_i$ values of 26 and 130 mM, respectively, for rabbit H4 and rabbit M4 reacting at 25° (ref. 4). Again there is some similarity between the plaice enzyme reacting at low temperature and the mammalian heart enzyme reacting at a higher temperature. Product inhibition data for beef H4 (LDH-1) at 10° are also shown in Fig. 4; at this temperature the beef H4



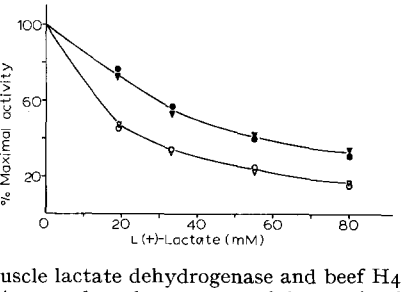




Fig. 3. Comparison of the effect of pyruvate on plaice muscle lactate dehydrogenase and beef H4 lactate dehydrogenase at 10°. The initial reaction velocities are plotted as percent of the maximal rate against the concentration of pyruvate. ▲, plaice muscle; ▽, beef H4. NADH concn., 0.15 mM; 0.1 M phosphate buffer (pH 7.4).

Fig. 4. Effect of product inhibition by L-lactate on pyruvate reduction by plaice muscle lactate dehydrogenase at 10°. The initial reaction velocity is plotted as percent of the uninhibited (maximal) rate against the concentration of L-lactate. Pyruvate concentrations were 0.1 mM (▽, ▼) and 0.046 mM (○, ●); NADH concn., 0.15 mM 0.1 M; phosphate buffer (pH 7.4). ▽, ○, beef H4; ●, ▼, plaice.

enzyme differs distinctly from plaice muscle lactate dehydrogenase and the H4 has a $K_i$ value of 17 mM.

## DISCUSSION

The low-temperature data reported here indicate that the plaice cardiac/skeletal muscle enzyme acting at 10° and the beef H4 acting at 35° have somewhat similar types of substrate and product inhibition. However the beef H4 reacting at 10° differs distinctly from the plaice enzyme in its inhibition characteristics.

In attempting to relate the findings on the plaice enzyme to current theories of lactate dehydrogenase isoenzyme function two factors must be borne in mind: (i) the extent of lactate dehydrogenase inhibition is affected by the particular assay conditions employed[17], *i.e.* these conditions play a part in determining the extent of inhibition encountered; (ii) functional differences are relative and evidence *in vitro* cannot be applied directly to events *in vivo*. Nevertheless the results reported here indicate that low temperature is an important factor affecting substrate and product inhibition of lactate dehydrogenase isoenzymes, as well as their absolute activity. How then does lactate dehydrogenase function effectively in the skeletal muscle of fish at these lower temperatures? The answer to this probably lies in the fact that fish lactate dehydrogenases generally have much higher turnover numbers than do mammalian and avian lactate dehydrogenases[8]. Moreover fish skeletal muscle appears to contain more lactate dehydrogenase than does comparable tissue from birds and mammals[8].

The second question concerns the presence of a predominantly M-type enzyme in the cardiac muscle of flatfish. Most other vertebrates appear to require the presence in the heart of a lactate dehydrogenase having markedly different properties from the skeletal muscle lactate dehydrogenase. It is difficult to comprehend why flatfish should be an exception to this generality. The answer may lie in the physiology of the flatfish heart about which little is known. As KAPLAN *et al.*[18] have developed their thesis, "the H-type enzyme would be found in muscles geared to perform sustained activity ..."; now flatfish lead a fairly static existence, they are inactive for long periods of time and there may not therefore be a very great difference in the metabolic activity of cardiac and skeletal muscle. In a mammal or pelagic fish there is probably a much greater difference in the turnover and development of metabolic energy between heart and skeletal muscles than is the case with flatfish. Thus not all the muscle fibres in the flatfish heart may be contracting at any one time and this again would result in a comparatively low overall rate of metabolic activity. As to what fraction of fibres are normally operative it is not possible to estimate but these speculations may point a way out of the dilemma of an M-type lactate dehydrogenase in the cardiac muscle of flatfish.

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REFERENCES

1 P. G. W. PLAGEMANN, K. F. GREGORY AND F. WRÓBLEWSKI, *Biochem. Z.*, 334 (1961) 37.
2 E. S. VESELL AND P. E. POOL, *Proc. Natl. Acad. Sci. U.S.*, 55 (1966) 756.
3 E. S. VESELL, *Ann. N.Y. Acad. Sci.*, 151 (1968) 5.
4 R. STAMBAUGH AND D. POST, *J. Biol. Chem.*, 241 (1966) 1462.
5 R. STAMBAUGH AND D. POST, *Biochim. Biophys. Acta*, 122 (1966) 541.
6 R. D. CAHN, N. O. KAPLAN, L. LEVINE AND E. ZWILLING, *Science*, 136 (1962) 962.
7 I. E. LUSH, C. B. COWEY AND D. KNOX, *J. Exptl. Zool.*, 170 (1969), in the press.
8 A. PESCE, T. P. FONDY, F. STOLZENBACH, F. CASTILLO AND N. O. KAPLAN, *J. Biol. Chem.*, 242 (1967) 2151.
9 M. D. POULIK, *Nature*, 180 (1957) 1477.
10 C. H. W. HIRS, *J. Biol. Chem.*, 219 (1956) 611.
11 S. MOORE AND W. H. STEIN, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 6, Academic Press, New York, 1963, p. 519.
12 D. C. THOMAS, in D. I. SCHMIDT, *Techniques in Amino Acid Analysis*, Technicon Instruments Co., Chertsey, Surrey, 1966, p. 116.
13 J. R. SPIES AND D. C. CHAMBERS, *Anal. Chem.*, 21 (1949) 1249.
14 A. J. PESCE, R. H. MCKAY, F. E. STOLZENBACH, R. D. CAHN AND N. O. KAPLAN, *J. Biol. Chem.*, 239 (1964) 1753.
15 N. O. KAPLAN, M. M. CIOTTI, M. HAMOLSKY AND R. E. BIEBER, *Science*, 131 (1960) 392.
16 T. P. FONDY AND N. O. KAPLAN, *Ann. N.Y. Acad. Sci.*, 119 (1965) 888.
17 E. S. VESELL, P. J. FRITZ AND E. L. WHITE, *Biochim. Biophys. Acta*, 159 (1968) 236.
18 N. O. KAPLAN, J. EVERSE AND J. ADMIRAAL, *Ann. N.Y. Acad. Sci.*, 151 (1968) 400.