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PREPARATIVE ISOLATION OF THE ISOENZYMES OF ADENOSINE DEAMINASE FROM BOVINE MUCOSA BY ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

1. A modified method, suitable for purifying larger quantities of adenosine deaminase (EC 3.5.4.4) is described.

2. Separation by high-voltage electrophoresis provides a rapid analytical method for detecting the isoenzymes.

3. The preparation in pure form of five isoenzymes by chromatography on Whatman DE 52 is described.

4. Deamination rate, K_m with adenosine and 6-chloropurine riboside, isoelectric point, denaturation by urea, heat stability, and inactivation by *p*-mercuribenzoate of the isoenzymes are compared.

INTRODUCTION

A large number of enzymes are now known to exist in multiple molecular forms. In the best characterised cases, isoenzymes have been shown to result from variation in the ratio of non-covalently linked inactive subunits, which differ in their amino acid composition[1].

Present evidence indicates that the isoenzymes of bovine adenosine deaminase (EC 3.5.4.4) cannot be accounted for on this basis because it appears to be a molecule composed of a single polypeptide chain of mol. wt. 34 000–35 000.

In the present paper the isolation of five of the six multiple forms present in mixed mucosa is described. A short account of portion of this work has been published[2].

METHODS AND RESULTS

In the present study it was necessary to use enzyme of specific activity greater than 400 units as less active preparations gave poor separation. A specific activity unit is defined as μmoles of adenosine deaminated per mg protein per min at pH 7 and 37°.

Modified purification of adenosine deaminase

When larger quantities of enzyme were processed by the original method[3], bacterial contamination frequently developed, and occasionally preparations with activities between 350–380 units, which could not be further purified by chromatography were encountered. A modified method which eliminates these problems, and allows the use of larger quantities of starting material, has been developed.

Extraction. 1% phenol which replaced aqueous extraction of acetone-dried mucosa was successful in suppressing bacterial contamination at later stages.

Acidification. 0.1 M HCl replaced satd. salicylic acid for acidification to pH 4.6 with a resultant decrease in fluid volume.

Fractional precipitation. Fractional precipitation with cold acetone of the original procedure[3] was retained unmodified.

The chromatographic procedure. The chromatographic procedure was extensively altered. DEAE-SF was replaced by Whatman DE-52 microgranular cellulose (H. Reeve Angel and Co., London). The dialysed protein solution from the acetone-precipitation stage was added to a column of DE-52, 28 cm × 1.5 cm previously equilibrated with 0.002 M sodium citrate buffer (pH 5.8). After washing off unabsorbed protein with this buffer, a concentration gradient to 0.033 M citrate (pH 5.8) was established. The enzyme eluted as a sharp peak with a specific activity between 340 and 360 units, in a yield of 40–47%. The pooled material, after overnight dialysis with three changes of distilled water, was concentrated by applying to a small column of DE-52, 5 cm × 1 cm and eluted in a volume of approx. 15 ml 2 M NaCl.

The eluate was added directly to a column of Sephadex G-75, fine, 59 cm × 2.5 cm and elution begun with 0.002 M sodium citrate buffer (pH 5.8). The enzyme eluted as a very sharp peak. In some purifications a small peak or shoulder with low activity emerged before the active enzyme peak. This material after concentration, was found to have a relative viscosity of 0.200, *i.e.* identical with urea-denatured adenosine deaminase and as urea-denatured adenosine deaminase elutes from a Sephadex G-75 column before the active enzyme this small peak may be denatured enzyme which is separated by the gel-filtration step.

200–350 mg of pure enzyme (400–470 units) can be prepared from 240 g of mucosa powder in a yield of 20–25% by this method.

Rapid method for detecting the isoenzymes of adenosine deaminase by high-voltage electrophoresis on cellulose acetate

In monitoring columns for their efficiency in separating the isoenzymes, and, for determining the most suitable regions of peaks to pool for single isoenzymes, large numbers of samples had to be assayed, so that a rapid method of isoenzyme analysis was required. High-voltage electrophoresis on cellulose acetate paper (Gelman Instrument Co., Ann Arbor, Mich., U.S.A.) proved successful. It gives excellent separation of the isoenzymes and the assay can be completed in 30 min.

Experimental procedure. A strip of cellulose acetate paper 20 cm × 5 cm was moistened thoroughly in 0.02 M phosphate buffer (pH 7.0), and excess liquid removed by blotting with absorbent paper. Samples were applied with a small platinum loop 4 cm from the cathode end at 1-cm intervals allowing the application of four samples with a 1-cm gap from each side of the paper. A further four samples were then applied 11 cm from the cathode end enabling eight samples to be assayed at a time.

Filter paper wicks were prepared from thin filter paper with a low water absorbancy. Post-lip filter paper (Technical Paper Sales, London) is suitable. Strips 60 cm × 7 cm were cut and folded to give a double wick 30 cm × 7 cm. After immersion in 0.02 M phosphate buffer, excess buffer had to be removed by vigorous blotting to eliminate liquid forming around the cellulose acetate during electrophoresis.

At the anode end, contact was made by placing the cellulose acetate strip between the two folds of the filter paper wick 1.5 cm from the end. When the same method was used at the cathode end charring was frequently encountered at the junction of the wick and the cellulose acetate paper, but by allowing the bottom flap of the wick to overlap by 2 cm and the top flap by only 1 cm this difficulty was overcome.

0.02 M phosphate buffer (pH 7.0) was used in the electrode compartments. Alternation of the pH between 6 and 8 had little effect upon the degree of separation.

The wicks and cellulose acetate paper were positioned on a large sheet of polythene on a Shandon high-voltage electrophoresis apparatus. A small section of polythene sheet 17 cm × 6 cm was placed on the cellulose acetate paper covering it entirely with the exception of the two sandwiches which the strip made with the wicks. A large sheet of polythene was then placed over the whole assemply in accordance with the manufacturers instructions and a pressure of 10–13 lb/inch^2 was applied to the plates. A voltage of 4000 V was found to give the best isoenzyme separation in the shortest time interval. With careful attention to the correct degree of moisture of cellulose acetate paper and wicks it was possible to maintain the current between 6–8 mA with no danger of charring, and the electrophoresis was completed in 15 min. Zymograms were developed by covering the cellulose acetate paper with a solution of agar containing adenosine and phenol violet as previously described[3]. The type of zymogram obtained is shown in Fig. 2.

Isolation of isoenzymes by ion-exchange chromatography

For the purpose of identification the isoenzymes have been numbered 1 to 6. The isoenzyme showing greatest anodal migration is called, No. 1 and the one showing least migration No. 6.

Satisfactory separation, in reasonable yield, of five isoenzymes has been obtained by ion-exchange chromatography on Whatman DE-52 cellulose, using a concave chloride gradient. It was not possible to isolate isoenzyme No. 1. This isoenzyme is frequently absent from preparations and is never present in more than trace amount as judged by the colour development in zymograms.

Preparation of gradient. A suitable gradient was obtained by connecting in series two 600-ml beakers of 8 cm internal diameter each containing 500 ml of 0.15 M NaCl to a 1500-ml beaker, of 11.6 cm internal diameter containing 1000 ml 0.002 M citrate buffer (pH 5.8) and provided with a magnetic stirrer. Connection was made by means of overhead siphons.

Chloride determination. The chloride concentration of the eluate was followed by the sensitive potentiometric method of SANDERSON[4], which required only 0.2 ml of a fraction for analysis.

Chromatography. A number of different molarities of chloride were investigated. Satisfactory separation was obtained with either 0.2 M or 0.15 M, but not with 0.1 M NaCl.

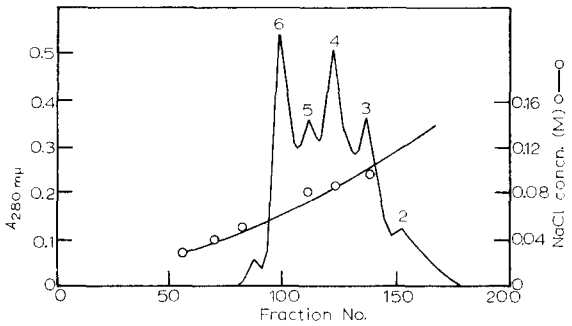

Fig. 1. Separation of isoenzymes of bovine adenosine deaminase by chromatography on Whatmann DE-52, using a concave gradient to 0.15 M NaCl. Experimental details are given in the text. The numbers 2 to 6 refer to the predominant isoenzyme present in the peaks.

The following is an account of a separation using 0.15 M NaCl. Chromatography was performed at 4°. 240 mg of purified adenosine deaminase of specific activity 418 units in 120 ml was added to a column of Whatman DE-52 micro-granular cellulose 28 cm × 1.5 cm previously equilibrated with 0.002 M sodium citrate buffer (pH 5.8). After absorption of the protein, the column was washed with 60 ml citrate buffer, and a concave gradient to 0.15 M NaCl in 0.002 M sodium citrate buffer established. The flow rate was 40 ml/h and 10-ml fractions were collected. The elution profile is shown in Fig. 1. Five peaks corresponding to the five isoenzymes were obtained. The individual fractions in the peaks were assayed by high-voltage electrophoresis and the fractions containing single isoenzymes were pooled and re-assayed. Zymograms of the collected fractions containing single isoenzymes are shown in Figs. 2a and 2b. The central zymogram in the figure shows the position of the isoenzymes in the enzyme preparation from which they were isolated and serves to identify them. It can be seen from examination of Figs. 1, 2a and 2b that the isoenzymes are eluted from the column in inverse relation to their migration on electrophoresis.

It will be noted that all of the isoenzymes have higher specific activities than the original enzyme, indicating a small further degree of purification during the separation. The differences in specific activity of the individual isoenzymes is not regarded as significant because the variability of the assay method is of the order of ± 10%.

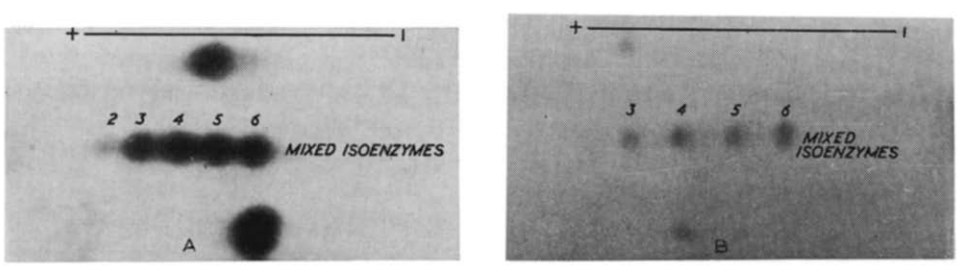

Fig. 2. Analysis of isoenzymes by high-voltage electrophoresis. Experimental details are given in the text. A. Pooled fractions containing single isoenzymes 5 and 6. B. Pooled fractions containing single isoenzymes 3 and 4. Isoenzyme 2 is not visible in the mixed isoenzymes in this photograph.

TABLE I

AMOUNT AND SPECIFIC ACTIVITY OF ISOENZYMES SEPARATED BY CHROMATOGRAPHY

Peak No.	*Vol. (ml)*	*Protein concn. (mg/ml)*	*Total protein (mg)*	*Specific activity (units)*	*Isoenzyme content (No.)*
6	68	0.47	32	487	6
5	47	0.39	18	449	5
4	90	0.496	44	461	4
3	61	0.343	21	449	3
2	40	0.107	4.3	489	2

Comparison of some properties of the individual isoenzymes

Deamination rate with adenosine, 2-deoxyadenosine and 3-deoxyadenosine (cordycepin) measured spectrophotometrically

10^{-4} M stock solutions of the three substrates were prepared in 0.1 M phosphate buffer (pH 7.0). For assay they were diluted to give a final molarity of $4 \cdot 10^{-5}$ M. The final molarity of each isoenzyme was 10^{-9} M based on a mol. wt. of 35 000. The experiments were performed in triplicate at 37°. 0.05 ml of the isoenzyme solution was added with mixing to 3 ml of substrate in a 1-cm cuvette and the fall in absorbance at 265 mμ was measured in a Beckman DU spectrophotometer provided with heating spacers. Taking the deamination rate of adenosine as unity the ratio with 2-deoxy- and 3-deoxyadenosine are given in Table II. It can be seen from Table II that the deamination rate of 2-deoxyadenosine by isoenzyme 4 was appreciably lower than with the other isoenzymes. The deamination rate of 3-deoxyadenosine was less than adenosine with all of the isoenzymes.

Calculation of K_m and v_{max} with adenosine and 6-chloropurine ribonucleoside as substrates

Preliminary studies showed that all of the isoenzymes had dechlorinase activity.

Adenosine. From a stock solution of $3.75 \cdot 10^{-4}$ M adenosine in 0.1 M phosphate buffer (pH 7.0), a range of dilutions between $1 \cdot 10^{-5}$ M and $1 \cdot 10^{-4}$ M were prepared for enzyme assay which was determined spectrophotometrically by the fall in absorbance at 265 mμ.

6-Chloropurine ribonucleoside. A stock solution of $1.5 \cdot 10^{-2}$ M 6-chloropurine ribonucleoside was made in 0.1 M phosphate buffer (pH 7.0) and a range of dilutions

TABLE II

COMPARISON OF DEAMINATION RATE OF THE ISOENZYMES

Isoenzyme No.	*Ratio deamination rates*	
	2-Deoxyadenosine	*3-Deoxyadenosine*
	Adenosine	*Adenosine*
6	1.08	0.66
5	0.97	0.70
4	0.86	0.83
3	1.09	0.71
2	0.98	0.818

TABLE III

K_m AND v_{max} VALUES OF THE ISOENZYMES WITH ADENOSINE AND 6-CHLOROPURINE RIBONUCLEOSIDE

Isoenzyme No.	*Adenosine*		*6-Chloropurine ribonucleoside*	
	v_{max}*	$K_m \times 10^5$ (*M*)	v_{max}*	$K_m \times 10^4$ (*M*)
6	374	2.8	531	10
5	300	1.5	154	2.5
4	—	—	149	2.08
3	550	1.5	105	1.0
2	330	1.7	375	1.51

* μmoles/min per mg protein.

from $3 \cdot 10^{-5}$ M to $1.26 \cdot 10^{-4}$ M prepared for enzyme assay. In this assay the rate of formation of inosine was measured by the increase in absorbance at 250 mμ (ref. 5).

The Michaelis constants for adenosine shown in Table III are lower than those reported in the literature for preparations containing a mixture of the isoenzymes where values between $3.3 \cdot 10^{-5}$ M (see ref. 6) and $8.3 \cdot 10^{-5}$ M (see ref. 5) have been found. The differences between the isoenzymes are small and are probably not significant. The calculated values for v_{max} are, with the exception of isoenzyme 3, 20–30% lower than the value determined directly in the presence of excess substrate. The calculated value for isoenzyme 3 is 20% higher.

The K_m values for 6-chloropurine ribonucleoside of isoenzymes 5, 4, 3, 2 are very similar and are approx. ten times greater than for adenosine, while the K_m of isoenzyme 6 is still higher. CORY AND SUHADOLNIK[5] reported a value of $6.4 \cdot 10^{-4}$ M for a preparation of the mixed isoenzymes.

The calculated v_{max} for the dechlorination reaction was highest with isoenzyme 6, while isoenzymes 5, 4, 3 were much lower and closer to the value of 100, obtained by CORY AND SUHADOLNIK[5] on a sample of the mixed isoenzymes. Isoenzyme 2 showed a rise in v_{max}. Sufficient substrate was not available for determinations in the presence of excess substrate.

Determination of isoelectric point

WILLS[7] has shown that many enzymes exhibit a pH-dependent inhibition of activity in the presence of Suramin, the mid-point of which coincides with their isoelectric point. A preparation of the mixed bovine enzyme has been shown to have an isoelectric point of 4.85 ± 0.05 by this method[3].

When the individual isoenzymes were tested all gave values between 4.8 and 4.9, *i.e.* their isoelectric points were, within the experimental limits of the method, identical. This is surprising in view of the differences in their electrophoretic mobilities which in the case of many isoenzymes is explained on the basis of charge differences.

Denaturation in 8 M urea

It has already been shown that the rate of denaturation of a preparation of the mixed isoenzymes was pH dependent[8]. As denaturation was minimal at pH 6.0 and rapid at pH 8, these two values were chosen to test if any difference could be detected in the susceptibility of the individual isoenzymes to urea denaturation.

Each isoenzyme, together with a sample of the mixed isoenzymes from which they were isolated (control) at a final concentration of $3 \cdot 10^{-6}$ M (0.1 mg/ml) were incubated at 37° in 8 M buffered urea (at pH 6.0 and 8.0). Enzyme activity was determined by microdiffusion at intervals up to 24 h.

The course of inactivation was plotted on a logarithmic scale against time on a linear scale, for the control and each isoenzyme. Although more dilute solutions of enzyme were used in this investigation the general pattern of results were similar to those shown in Fig. 2 of ref. 8.

After 24-h incubation at pH 6.0 the control had retained 68%; isoenzyme 6, 74%; isoenzyme 5, 70%; isoenzyme 4, 66%; isoenzyme 3, 64% and isoenzyme 2, 67% of their initial activity.

At pH 8.0 the control and isoenzymes showed first order kinetics for 5 h with a loss of 91–93% activity after which the rate decreased. No differences could be detected in the rate of denaturation of the individual isoenzymes.

Small differences in the stability of the isoenzymes are evident at pH 6.0. There is a progressive decrease in stability from isoenzyme 6 to isoenzyme 3 but isoenzyme 2 shows an increase with a stability similar to that of isoenzyme 4 and the mixed preparation. With the more rapid denaturation at pH 8, these differences are not apparent.

Inhibition by p-hydroxymercuribenzoate

p-Hydroxymercuribenzoate is known to inhibit the activity of adenosine deaminase by reacting with a sulphydryl group in the molecule[9]. As other sulphydryl reagents such as iodoacetate, and *N*-ethylmaleimide are not inhibitory the sulphydryl group necessary for activity is not highly reactive. It was, therefore of interest to test if differences in sensitivity to this mercurial could be detected in the individual isoenzymes.

Solutions of the isoenzymes containing 0.007 mg/ml protein ($2 \cdot 10^{-7}$ M) in 0.1 M sodium citrate buffer (pH 6.0) were incubated for 30 min at 37° with final concentrations of 10^{-5} M, $2.5 \cdot 10^{-5}$ M, $8.3 \cdot 10^{-5}$ M and 10^{-4} M *p*-mercuribenzoate and their activity compared with solutions of the isoenzymes without added inhibitor. While small differences were found at some concentrations they were not consistent throughout the concentration range and at the highest concentration of 10^{-4} M *p*-mercuribenzoate the isoenzymes were all inhibited to the extent of 90–95%. Activation by *p*-mercuribenzoate as observed by HOAGLAND AND FISHER[10] with chicken duodenal adenosine deaminase was not evident, but the experimental conditions were different and the transitory activation observed by these authors at $7.0 \cdot 10^{-5}$ M *p*-mercuribenzoate would not be detectable after 30 min incubation, as used in the present study.

Heat inactivation

The stability of the isoenzymes were tested at 60° and 70°. 2 ml of solutions containing 0.1 mg/ml of protein in 0.1 M phosphate buffer (pH 7.0) were heated in a constant temperature bath to 60° (70°) for 20 min and then rapidly cooled in iced water. Duplicate experiments were performed on each sample and activity was determined by microdiffusion. A preparation containing the mixed isoenzymes was included.

It can be seen from Table IV that isoenzyme 2 is more stable to heat than the other isoenzymes. At 60° it gives no fall in activity while the other isoenzymes and

TABLE IV

COMPARISON OF THERMAL STABILITY OF ISOENZYMES

Isoenzyme No.	*Inactivation (%)*	
	at 60°	*at 70°*
6	13	81
5	11	74
4	13	77
3	15	76
2	0	70
Mixed isoenzymes	13	79

the mixed preparation are inactivated to the extent of 11–15%. At 70° although the difference is less clear-cut isoenzyme 2 again shows the smallest degree of inactivation.

Structure of adenosine deaminase

Present evidence indicates that bovine adenosine deaminase does not possess a subunit structure. The enzyme is homogeneous in the ultracentrifuge[3] and gives a symmetrical peak of mol. wt. 35 000 on a column of standardised Sephadex G-75 (ref. 8). Enzyme denatured with 8 M urea, and with 2 M guanidine eluted from Sephadex G-75 columns as single symmetrical peaks of apparently higher mol. wt. than the native enzyme. Performate oxidized enzyme was also retarded but emerged as a single peak with no evidence of smaller fragments. Neither sulphite-treated enzyme, used to cleave the disulphide bonds, nor urea-denatured carboxymethylated enzyme showed evidence of release of smaller fragments on elution from Sephadex columns[13]. As the N-terminal group is not free it has not been possible to determine it, but only one carboxyterminal amino acid, valine, has been detected by carboxypeptidase A which supports the view that the enzyme is composed of a single polypeptide chain.

DISCUSSION

While many enzymes have been resolved into isoenzymes, the differences at molecular level, which result in these multiple forms are known in only a few cases. It is possible that some multiple forms may prove to be artifacts produced as a result of enzymatic or other action, after the cell dies or during the isolation or purification of the enzyme.

These possibilities have been considered in the case of the multiple forms of adenosine deaminase, and while proof of the existence of isoenzymes within the cell is lacking, certain evidence has been obtained suggesting that some of the multiple forms are true isoenzymes. For instance extracts prepared from the mixed mucosa derived from 10 to 20 animals invariably have five and occasionally six isoenzymes but never more. In numerous purifications from such extracts isoenzymes were neither gained nor lost and their relative concentrations, as judged by zymograms, remained unchanged. Incubation of these extracts and of purified enzyme for 48 h at 37° with the proteolytic enzymes trypsin, chymotrypsin, carboxypeptidase, leucine amino-

peptidase, subtilisin, fibrinolysin, ficin and papain caused neither increase nor decrease in the number of isoenzymes[11]. It is clear that the isoenzymes are not the result of preparative procedures, at least after the initial step, the isolation of the enzyme from the cell, and they are unaffected by a large number of proteolytic enzymes.

A different picture emerged when the mucosa of single animals were examined immediately after death. In this case usually only two isoenzymes were present but their electrophoretic position varied from animal to animal. The five- to six-isoenzyme pattern found in mixed mucosa appears to be a fortuitous result of the mixing of the two-isoenzyme pattern present in single mucosa, and this is supported by the observed variation in the amount of different isoenzymes which have been isolated from different batches of mucosa by chromatography.

A further complication in the interpretation of the isoenzymes arises from the fact that any form of storage of single mucosa, either frozen, at 4°, room temperature, or at 37° leads to an increase in the number of multiple forms from two to four or more. It is unlikely that the increase is due to enzyme action because the rate of increase is the same in frozen extracts as it is in extracts held at higher temperatures. Furthermore addition of certain sulphydryl reagents such as iodoacetamide, *N*-ethylmaleimide and also mercaptoethanol reduces the formation of isoenzymes on storage, and if added after their formation leads to a reduction in their number.

An increase in the number of multiple forms has been observed by SPENCER, HOPKINSON AND HARRIS[12] in human serum adenosine deaminase. They suggest that it may be the result of exchange reactions between disulphides in the serum and a reactive sulphydryl group in the enzyme. The bovine enzyme is known to have two sulphydryl groups one of which is essential for enzyme activity[9]. It is quite possible that sulphydryl exchange reactions as postulated by SPENCER, HOPKINSON AND HARRIS[12] may also account for the increase observed with the bovine enzyme. Addition of mercaptoethanol to purified enzyme prepared from mixed mucosa does not cause any change in the number of isoenzymes. There do, therefore, appear to be two types of multiple forms of adenosine deaminase, true isoenzymes, and artifacts produced by some form of interaction with other cell constituents. These latter are, at present, being investigated.

The stable multiple forms which have been isolated are very similar in properties, and no systematic variations from the slowest migrating No. 6 to the fastest No. 2 have been detected.

Some preliminary structural studies have not revealed any differences. The N-terminal group is substituted in each of the isoenzymes and tryptic digests separated by one dimensional high-voltage electrophoresis have not revealed any significant differences in the number and type of peptides released.

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REFERENCES

1 H. E. AEBI, *Ann. Rev. Biochem.*, 36 (1967) 279.
2 T. G. BRADY, P. M. MURPHY AND P. COLLINS, *Proc. 7th Intern. Congr. Biochem. Tokyo Abstr.*, IV (1967) F 123.
3 T. G. BRADY AND W. O'CONNELL, *Biochim. Biophys. Acta*, 62 (1962) 216.
4 P. H. SANDERSON, *Biochem. J.*, 52 (1952) 502.
5 J. G. CORY AND R. J. SUHADOLNIK, *Biochemistry*, 4 (1965) 1733.
6 S. FREDERIKSEN, *Arch. Biochem. Biophys.*, 113 (1966) 383.
7 E. D. WILLS, *Biochem. J.*, 50 (1952) 421.
8 T. G. BRADY AND M. O'SULLIVAN, *Biochim. Biophys. Acta*, 132 (1967) 127.
9 G. RONCA, C. BAUER AND C. A. ROSSI, *European J. Biochem.*, 1 (1967) 434.
10 V. D. HOAGLAND AND J. R. FISHER, *J. Biol. Chem.*, 242 (1967) 4341.
11 M. NOONAN, M. Sc. Thesis, National University of Ireland, 1967, p. 13.
12 N. SPENCER, D. A. HOPKINSON AND H. HARRIS, *Ann. Human Genet.*, 1968, in the press.
13 J. PHELAN, Ph. D. Thesis, National University of Ireland, 1966, p. 227.