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Separation and Characterization of Two Rat-Intestinal Amylases

By A. DAHLQVIST and D. L. THOMSON
Department of Physiological Chemistry, University of Lund, Lund, Sweden

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During a recent study of the carbohydrases of rat-intestinal mucosa, the amylase activity was found to yield two separate peaks on ion-exchange chromatography (Dahlqvist, 1963). This paper deals with the characterization of the two enzymes responsible for the amylase activity of these extracts.

MATERIALS AND METHODS

Determination of carbohydrase activities. The amylase activity was measured by the method described by Dahlqvist (1961*a*). One unit of amylase activity is now defined as the activity releasing reducing groups corresponding to 1 μmole of maltose/min. at 37° (Dahlqvist, 1963). The disaccharidase activities were assayed with a tris-buffered glucose-oxidase reagent (Dahlqvist, 1961*b*), under the same incubation conditions as those described for the pig enzymes (Dahlqvist, 1960, 1961*b*). One unit of disaccharidase activity is defined as the activity hydrolysing 1 μmole of disaccharide/min. at 37°.

Determination of protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with the modified 'reagent B' introduced by Eggstein & Kreutz (1955). A standard curve was prepared with freshly dissolved human serum albumin, kindly supplied by AB Kabi (Stockholm, Sweden).

Thin-layer chromatography. The method of Weill & Hanke (1962), using a solvent mixture containing butan-1-ol–2,6-lutidine–water (6:3:1, by vol.), was used. Glucose and maltose (5 μg. each) were used as markers, and 10 μl. of the reaction mixture for amylase determination was applied to the plate after the reaction had proceeded for the desired time at 37° and had been interrupted by placing the tube in boiling water for 2 min.

Viscosimetry. The soluble starch preparation used for the reduction method of assay of amylase (Dahlqvist, 1961*a*) yields a solution with very low viscosity and is therefore not suitable for viscosimetric studies. For this purpose a solution prepared from commercial rice starch was used instead. The substrate solution was prepared by suspending 2·0 g. of rice starch in 100 ml. of 0·1 M-phosphate-buffered NaCl at pH 6·9 (3·03 g. of KH_2PO_4, 3·96 g. of $Na_2HPO_4,2H_2O$ and 0·400 g. of NaCl diluted with water to 1000 ml.). The suspension was placed in a boiling-water bath for 15 min. with automatic stirring, and left to cool. A suitably diluted enzyme solution was incubated at 37° with an equal volume of the buffered substrate and the reaction stopped by mixing 5 ml. of the reaction mixture with 0·5 ml. of 1 N-acetic acid. The viscosity was determined in an Ostwald viscosimeter and the relative viscosity (viscosity/viscosity of water) calculated. With the same substrate, the liberation of reducing groups was determined with the 3,5-dinitrosalicylate reagent of Sumner (1924), as in the usual assay procedure for amylase (Dahlqvist, 1961*a*). The reaction was stopped by heating the tubes in boiling water for 2 min.

Animals. Sprague–Dawley rats of both sexes, weighing 150–200 g., were used.

Mucosal homogenates. The rats were starved for 12–18 hr., with free access to water, and then killed by a blow on the head. The proximal two-thirds of the small intestine was removed and immediately placed in ice-cold 0·9% NaCl. The intestine was then cut open, rinsed with chilled 0·9% NaCl, blotted gently and the mucosa scraped off with a glass slide. After weighing, the mucosa was mixed (1 g./4 ml.) with ice-cold 0·2 M-sodium phosphate or 0·1 M-sodium maleate buffer, pH 7·0. Several drops of octan-2-ol were added to prevent foaming and the mixture was homogenized for 2 min. with an Ultra-Turrax homogenizer.

Table 1. *Distribution of amylase, disaccharidases and protein in a crude homogenate of rat intestinal mucosa*

Six rats were used. Experimental details are given in the text.

Preparation	Amylase (units/ml.)	Maltase (units/ml.)	Isomaltase (unit/ml.)	Invertase (unit/ml.)	Protein (mg./ml.)
Crude mucosal homogenate	82	2·26	0·60	0·41	13·6
Soluble enzymes	63·9	0·41	0·05	0·02	8·7
Washed particles	1·27	1·50	0·53	0·42	2·2

Table 2. *Effect of proteolytic enzymes and autolysis on the solubilization of the amylase and maltase of the 'washed-particle' fraction of homogenates of rat intestinal mucosa*

Experimental details are given in the text. The values represent the percentage of the original amount of activity found after the stated treatment and the percentage of the original activity in the supernatant after centrifuging at 100000*g* for 1 hr.

Treatment	Amylase remaining (%)	Soluble amylase (%)	Maltase remaining (%)	Soluble maltase (%)
Starting material	100	11	100	21
Papain digestion (1 hr.)	64	61	100	87
Trypsin digestion (1 hr.)	42	19	91	74
Ficin digestion (1 hr.)	84	29	91	42
Autolysis (1 hr.)	77	16	83	31

The tube was chilled with crushed ice during the homogenization. The homogenate was then centrifuged at 1000*g* for 10 min. in an International Refrigerated Centrifuge, model HR-1, and the opalescent supernatant fluid used as the crude mucosal homogenate. If not used at once, the homogenates were stored at −16°.

Soluble enzymes. The crude homogenate was centrifuged at 100000*g* for 60 min. at 4° in a Spinco preparative ultracentrifuge. The supernatant fluid was used to assay the soluble enzymes.

Washed particles from mucosal homogenates. The sediment after the centrifuging of the crude homogenate at 100000*g* was resuspended in ice-cold water to give the original volume of the homogenate. Suspension was performed by hand with a homogenizer of the Potter & Elvehjem (1936) type with a loose-fitting glass pestle, and the suspension was again centrifuged at 100000*g* for 1 hr. The particles were washed in the same manner a second time and then resuspended in 0·1 M-potassium phosphate buffer, pH 7·0, to give the original volume of the homogenate.

Solubilization of amylase and maltase from washed particles. A portion (0·2 ml.) of a preparation of washed particles in 0·1 M-potassium phosphate buffer, pH 7·0, was incubated for 1 hr. at 37° with 0·2 ml. of one of the following solutions: crystalline trypsin (Novo Industri A/S, Copenhagen, Denmark), 0·1 mg./ml.; twice-crystallized papain (Sigma Chemical Co.), 0·25 mg./ml., activated by 0·5 mg. of cysteine hydrochloride/ml.; twice-crystallized ficin (Sigma Chemical Co.), 0·1 mg./ml. As a control for the effect of autolysis, 0·2 ml. of the preparation was also incubated with 0·2 ml. of water at 37° for 1 hr. After incubation the tubes were placed in crushed ice and the contents diluted to 10 ml. with water. Portions (2 ml.) of the diluted solutions were withdrawn for analysis of the total amylase and maltase activity left after protease digestion, and the remainder was centrifuged at 100000*g* for 60 min. The supernatant was analysed for the solubilized enzymes.

Enzyme-fractionation methods. Heat-inactivation of the enzyme activities and anion-exchange chromatography on triethylaminoethylcellulose (TEAE-cellulose) with salt-gradient elution were performed as described by Dahlqvist (1959, 1963).

RESULTS

Activity of the crude mucosal homogenates. The crude mucosal homogenates, prepared as described above, contained (in each ml.) 71·2–93·5 units of amylase, 1·80–2·75 units of maltase, 0·59–0·61 unit of isomaltase, 0·31–0·49 unit of invertase and 11·62–16·75 mg. of protein.

Solubility of the carbohydrase activities. Most of the amylase was soluble and was obtained in the supernatant fluid after the first ultracentrifuging. Less than 2% of the total amylase activity of the crude homogenates was recovered in the washed particles (Table 1). Most of the disaccharidase activity was in the particles. The distribution of the carbohydrase activities was similar in five separate experiments, with a total of 20 rats.

Solubilization of the particulate amylase and maltase. Of the treatments used, only papain digestion resulted in the solubilization of more than 50% of both the amylase and maltase from the washed particles (Table 2). When a mucosal homogenate prepared in maleate buffer as described in the Materials and Methods section was treated with the same concentration of papain used for the particles and incubated at 37° for 60 min., 73% of the amylase and 98% of the maltase was found in the supernatant fluid after ultracentrifuging at 100000*g*. Trypsin destroyed a considerable amount of the amylase in both the washed particles and crude homogenates, whereas ficin digestion or autolysis resulted in much less solubilization of the enzymes than was observed with papain.

Anion-exchange chromatography. When a papain-solubilized total homogenate was chromatographed on TEAE-cellulose over 95% of the amylase activity occurred in an early peak (amylase A), at which time the buffer concentration was 0·01 M (Fig. 1). The second peak (amylase B) was widely separated from the first and much smaller. The elution of amylase B did not occur until the buffer concentration exceeded 0·06 M. Of the amylase activity applied to the column 80% was recovered in the eluate.

Two amylase peaks were also found when the papain-solubilized enzymes from the washed particles were chromatographed (Fig. 2). The amylase B activity, which in this case amounted to more than 50% of the total amylase activity, closely followed the third maltase peak and was separated from the isomaltase and invertase activities.

Larger amounts of amylase A and B were prepared by stepwise elution of the two enzymes from columns containing 5·0 g. of TEAE-cellulose, after application of papain-solubilized crude homogenates to the column. Amylase A was eluted with 0·01 M-sodium phosphate buffer, pH 6·0, the column was eluted with 0·07 M-buffer until no amylase activity could be detected in the effluent and amylase B was then eluted with 0·14 M buffer. The isolated amylases A and B prepared in this way were used for the study of their individual properties.

From the chromatographic and centrifugal findings it was concluded that over 95% of the amylase activity of the crude mucosal homogenates was due to amylase A and less than 5% due to amylase B.

Effect of dialysis. When a crude mucosal homogenate in 0·2 M-sodium phosphate buffer was dialysed for 16–18 hr. at 4° against 0·01 M-sodium phosphate buffer, pH 6·0, a considerable loss of amylase activity occurred. After dialysis, less than 15% of the original amylase activity was recovered. On addition of calcium chloride to the dialysed solution, a partial reactivation of the amylase was obtained but the recovery was still less than 25% of the original amylase activity. The irreversible loss may be explained by the presence of proteolytic enzymes in the homogenates that are able to

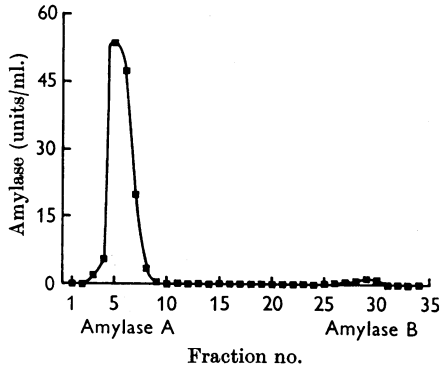

Fig. 1. Gradient-elution chromatography on TEAE-cellulose of papain-solubilized amylases from homogenate of rat-intestinal mucosa. The mucosal homogenate was prepared in 0·1 M-sodium maleate buffer, pH 6·0, as described in the text and shaken with 0·25 mg. of papain and 0·5 mg. of cysteine hydrochloride/ml. at 37° for 60 min. The preparation was then ultracentrifuged at 100000*g* for 60 min. and the supernatant fluid dialysed against 1 mM-$CaCl_2$ at 4° for 18 hr. A portion (15 ml.) of the dialysis residue in 0·01 M-sodium phosphate buffer, pH 6·0, containing 516 units of amylase, was applied to a column (10 mm. × 55 mm.) containing 0·5 g. of TEAE-cellulose. Elution was performed as described by Dahlqvist (1963). Each fraction had a volume of 3·2 ml.

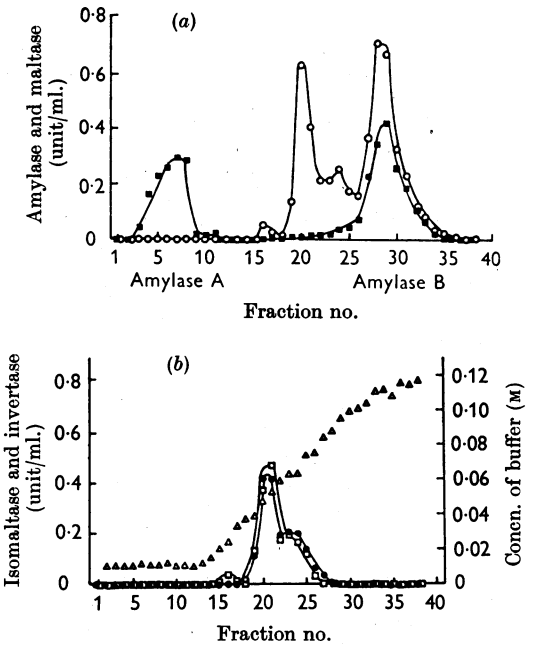

Fig. 2. Gradient-elution chromatography of papain-solubilized carbohydrases from washed particles from rat-intestinal mucosa. Details of the preparation and solubilization of the enzymes are given in the text. The supernatant after papain-digestion and ultracentrifuging was dialysed against 0·01 M-sodium phosphate buffer, pH 6·0, at 4° for 18 hr. A portion (18·5 ml.) of the dialysis residue containing 7·02 mg. of protein, 12·0 units of amylase, 15·9 units of maltase, 7·76 units of isomaltase and 5·18 units of invertase was applied to a column containing 0·5 g. of TEAE-cellulose. The column was prepared and run as described in Fig. 1. The volume of each fraction was 3·0 ml. (*a*) ■, Amylase; ○, maltase. (*b*) □, Invertase; ●, isomaltase; △, buffer concentration.

destroy certain amylases when Ca^{2+} ions are removed (Stein & Fischer, 1958). The disaccharidase activities were not decreased by dialysis.

When crude mucosal homogenates in 0·1 M-sodium maleate buffer were dialysed against 1 mM-calcium chloride more than 60 % of the original amylase activity was recovered in the dialysis residue.

Isolated amylase A, when dialysed against phosphate buffer, showed as great a loss of activity as did the crude homogenate; more than 90 % of the activity of isolated amylase B was recovered after this procedure.

Properties of the isolated amylases

Specificity. Amylase A had no detectable maltase, isomaltase or invertase activity (less than 0·01 unit/ml. in a preparation containing 54 units of amylase A/ml.). Amylase B showed a maltase activity of 95–110 % of its amylase activity, but no detectable invertase or isomaltase.

Influence of pH. Amylase B had a more acid pH optimum than amylase A (Fig. 3*a*). The maltase activity of amylase B, tested with the same buffers as used for the amylase activity, showed an optimum from pH 6·0 to 7·0 (Fig. 3*b*).

Effect of Cl^- *ions.* Amylase A is activated by Cl^- ions (Table 3). The maltase and amylase activities of amylase B do not show this effect.

Reaction products. (*a*) Thin-layer chromatography. The products of the action of amylase A and B acting on a 1 % soluble starch solution were examined by thin-layer chromatography as described in the Materials and Methods section. With amylase A, 0·028 mg. of protein was present in 2 ml. of reaction mixture, and with amylase B there was 0·022 mg. of protein in 2 ml., 0·24 unit of amylase activity being present in each case. No glucose was detected when the reaction products of amylase A were followed for 1 hr. Maltose and a series of higher oligosaccharides were present after 10 min. and increased in amount up to 60 min. In the reaction mixtures containing amylase B, glucose was clearly detected after 5 min. and was the only reaction product for the first 30 min. After 45 and 60 min. a weak spot of maltose was also seen.

(*b*) Chemical determination. Chemical analysis of the reaction products agreed well with the findings on thin-layer chromatography (Table 4). Amylase A did not produce a measurable amount of glucose and amylase B produced glucose to account for virtually all of the reducing groups liberated during the first 10 min. of the reaction.

Heat-inactivation. Amylase A was considerably more sensitive to heat than amylase B. When heated at 50° in 0·01 M-sodium phosphate buffer, pH 6·0, over 90 % of the amylase A was inactivated in 20 min. Amylase B was not measurably affected when heated at 50° in the same buffer for the same length of time. When a similar solution of amylase B was heated at various temperatures between 54° and 60° a close parallel between the inactivation of its amylase and maltase activities was observed. This strongly suggests that both of these activities were exerted by the same protein.

Viscosity changes during amylase action. When a solution containing 0·25 unit of amylase A/ml. (measured by the ordinary method) acted on a

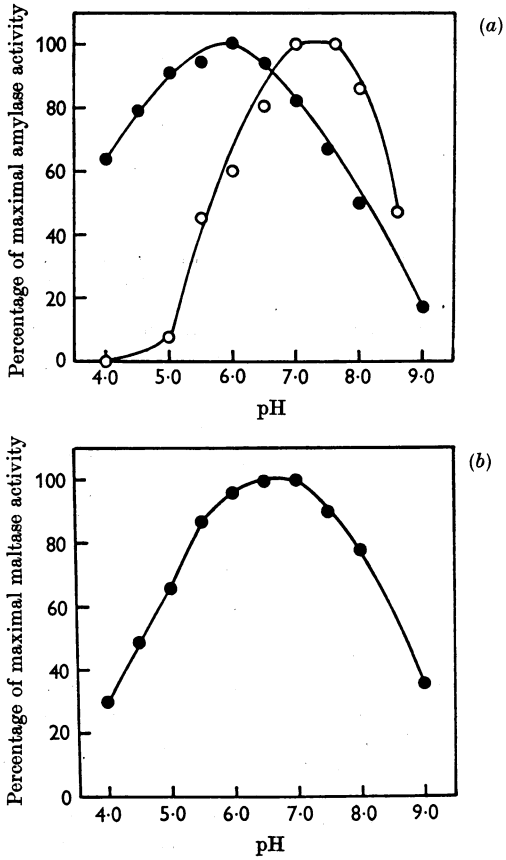

Fig. 3. Influence of pH on the activity (*a*) of the two isolated rat-intestinal mucosal amylases and (*b*) of the maltase of amylase B. (*a*) Reaction mixture contained 0·004 mg. of protein for amylase A assay, and 0·032 mg. of protein for amylase B assay, and was the same as that used in the usual assay method for amylase (Dahlqvist, 1961*a*) except for the buffer and pH. Buffers used were: pH 4·0–5·0, 0·05 M-sodium acetate; pH 5·5–6·0, 0·05 M-sodium maleate; pH 6·5–7·0, 0·05 M-sodium phosphate; pH 7·6–8·6, 0·05 M-sodium–tris maleate for amylase A, and pH 7·5–9·0, 25 mM-sodium veronal for amylase B. (*b*) Reaction mixture for the assay of the maltase activity of amylase B contained 0·00075 mg. of protein and was the same as that of the usual assay method except for the buffer and pH. ○, Amylase A; ●, amylase B.

Table 3. *Influence of* Cl^- *ions on amylase A and B*

For the assay of amylase activity the reaction mixture contained 0·05M-sodium maleate buffer, pH 6·5, 1% soluble starch (Zulkowski) and 0·003 mg. of protein for amylase A, or 0·006 mg. of protein for amylase B, in 2·0 ml. The concentration of NaCl in the reaction mixture was varied as indicated. To assay the maltase activity of amylase B the mixture contained 28 mM-maltose instead of starch. The liberation of reducing groups from starch was determined with the 3,5-dinitrosalicylate reagent of Sumner (1924). The liberation of glucose from maltose was determined with a glucose-oxidase reagent containing tris (Dahlqvist, 1961*b*).

Amylase A		Amylase B		
Concn. of NaCl (mM)	Starch substrate (mg. of 'maltose' formed in 60 min. at 37°)	Concn. of NaCl (mM)	Starch substrate (mg. of 'maltose' formed in 60 min. at 37°)	Maltose substrate (mg. of 'glucose' formed in 60 min. at 37°)
0	0·11	0	0·73	0·48
1	0·64	1	0·82	0·49
2	0·70	2·5	0·83	—
4	0·76	5	0·78	0·50
6	0·76	10	0·84	0·49
8	0·81	100	0·80	—
10	0·82	—	—	—

Table 4. *Chemical determination of the products of the action of amylase A and B on starch*

The reaction mixture was the usual one for the determination of amylase activity (Dahlqvist, 1961*a*). For amylase A 0·004 mg. of protein, and for amylase B 0·032 mg. of protein, was present in 2 ml. of reaction mixture. The liberation of reducing groups was determined with the 3,5-dinitrosalicylate reagent with glucose as a standard and this reagent was used to stop the reaction after the desired time. Glucose was determined at 37° with glucose oxidase (Dahlqvist, 1961*b*), after the amylase had been destroyed by heating in boiling water for 2 min.

	Amylase A		Amylase B	
Incubation time (min.)	Reducing groups liberated (mg.)	Glucose formed (mg.)	Reducing groups liberated (mg.)	Glucose formed (mg.)
5	1·33	0	0·12	0·16
10	2·13	0	0·28	0·24
20	4·39	0	0·62	0·44
30	6·25	0	0·96	0·60
45	8·63	0	1·42	0·94
60	11·17	0	1·90	1·16

solution of rice starch, prepared as described above, the relative viscosity decreased from 1·58 at zero time to 1·27 in 10 min. and to 1·15 in 30 min. This corresponds to approximately 50 and 75% hydrolysis respectively, as measured by this method. As calculated from the increase in reducing power, however, only 0·6% of the glucosyl links had been hydrolysed after 30 min.

An equal amount (units/ml.) of amylase B did not produce a measurable decrease in viscosity during 30 min., and 0·6% of the starch was hydrolysed as determined by the increase in reducing power.

Effect of EDTA. In the presence of 1 mM-EDTA, amylase A was 95% inactivated and amylase B was little affected.

DISCUSSION

Since rat pancreatic juice has very high amylase activity, the possibility exists that some part of the amylase activity in the intestinal mucosal homogenates originates from the pancreas and has not been removed by washing the mucosa with 0·9% sodium chloride solution. However, it has been found that rats can digest starch after the removal of the pancreas and salivary glands (McGeachin, Gleason & Adams, 1958), and the small-intestinal mucosa of these rats had a high amylase activity (McGeachin & Ford, 1959). Therefore a considerable amount of amylase seems to be formed in the small-intestinal mucosa itself. The presence of both of the amylases we have studied in the washed particles from the mucosal homogenates supports the assumption that they are produced in the mucosa.

Amylase A is responsible for the major part of the amylase activity of the mucosal homogenates. It has the properties of an endoamylase (α-amylase), hydrolysing starch to maltose and higher oligosaccharides. The random hydrolysis of the α-(1→4)-glucosidic linkages in the starch molecules accounts for the rapid decrease in viscosity

of the rice-starch solutions. This enzyme is activated by Cl^- ions, inhibited by EDTA and does not hydrolyse disaccharides.

Amylase B is responsible for only a small fraction of the total amylase activity of the mucosa, but accounts for about half of the amylase activity of the washed particles. It has the properties of a glucamylase (γ-amylase; for a review see Larner, 1960). Glucose is the primary reaction product of the action of a glucamylase on starch. Glucamylases are exoamylases, hydrolysing the terminal α-(1→4)-glucosidic linkages at the non-reducing end of starch chains. During its action the viscosity of a starch solution decreases very slowly. Amylase B also hydrolyses maltose to glucose at approximately the same rate as starch is hydrolysed. It is responsible for about half of the maltase activity of the mucosal preparations.

The physiological function of the intestinal glucamylase cannot be stated at present. Glucamylases have been demonstrated in mammalian liver (Glock, 1936; Torres & Olavarria, 1961; Rosenfeld & Popova, 1962; Lejeune, Thinès-Sempoux & Hers, 1963) and muscle (Torres & Olavarria, 1961; Hers, 1963).

SUMMARY

1. Rat small-intestinal mucosa contains two amylases, one an endoamylase (α-amylase) and one an exoamylase (glucamylase, γ-amylase).

2. The endoamylase accounts for the major part of the total amylase activity of the mucosa. It is chiefly present in a soluble form in mucosal homogenates and has no maltase activity.

3. The glucamylase is chiefly particle-bound in mucosal homogenates. It can be solubilized by papain-digestion of the particles. This enzyme hydrolyses maltose at approximately the same rate as starch. It accounts for about half of the total maltase activity of the mucosa. It has no isomaltase or invertase activity.

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Measurement of Synthesis Rates of Liver-Produced Plasma Proteins

By A. S. McFARLANE
National Institute for Medical Research, London, N.W. 7

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Hitherto rates of synthesis of plasma proteins have been inferred from measurements of catabolism in man and animals in protein equilibrium, the important clinical problem of measuring differences between synthesis and catabolic rates in unbalanced states being as yet unresolved. The problem of measuring protein-synthesis rates absolutely, as in nearly all tracer problems, is that of measuring the specific activities of intracellular precursor amino acids. If a mean value for these