preference for tyrosine, it acts less readily on *m*-hydroxyphenylalanine and has practically no affinity for *o*-hydroxyphenylalanine.

The bacterial and the mammalian enzyme are closely related; both are pyridoxalphosphate-proteins (Gunsalus, Bellamy & Umbreit, 1944; Gale, 1946; Green, Leloir & Nocito, 1945; Blaschko, Carter, O'Brien & Sloane Stanley, 1948). Their different affinities for tyrosine and its two isomers must, therefore, be due to structural differences of the enzyme proteins. The observations reported here show that the position of the phenolic hydroxyl group is one of the factors which govern the enzyme-substrate relationships of the two decarboxylases.

SUMMARY

1. Extracts of rat liver and guinea pig kidney form carbon dioxide when incubated anaerobically with *o*-hydroxyphenylalanine; it is suggested that *L*-*o*-hydroxyphenylalanine is a substrate of DOPA decarboxylase.

2. *o*-Hydroxyphenylalanine is a substrate of the *D*-amino-acid oxidase of pig kidney.

3. *o*-Hydroxyphenylalanine is a substrate of the *L*-amino-acid oxidase of cobra venom.

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Reducing-group Production from Starch by the Action of α - and β -Amylases of Barley Malt. Activity of α - and β -Amylases

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Recent proposals for the determination of α -amylase of barley malt (Graesser & Dax, 1946; Preece, 1947, 1948) involve differential heat inactivation at 70° in the presence of calcium ions, followed by standardized conversion of a soluble starch substrate (American Society of Brewing Chemists, 1944; Institute of Brewing, 1933), with determination of reducing groups by ferricyanide titration or by Fehling's titration respectively. Thus, β -amylase is heat-inactivated and the observed saccharification of the starch is brought about by α -amylase alone (cf. Kneen, Sandstedt & Hollenbeck, 1943). Total saccharification by the joint action of the α - and β -amylases present in the malt being readily determined as a measure of diastatic power by a similar method with the heat inactivation omitted, saccharification by β -amylase should be obtainable by difference.

These proposals involve a number of assumptions. Thus, it is necessary to be able to work under such conditions for starch conversion that the production of reducing groups shows a linear relationship with enzyme concentration and with duration of action, and also to know that the reactions of the two components are, in fact, truly additive in the reaction range used. The published data relating to these points have been developed under a wide variety of experimental conditions. Thus, of the more recent work, Kneen & Sandstedt (1941), using 2% (w/v) soluble starch solution for hydrolysis at 30°, find linearity between enzyme concentration and reducing-group production (ferricyanide method) up to 40% hydrolysis for the joint action of the two enzymes, and up to 30% for β -amylase alone; for α -amylase they found no region of linearity. Further, it was shown that, under the experimental condi-

tions used, saccharification by the two components was additive for a wide range of ratios of β - to α -amylase saccharogenic activity, deviating slightly, however, when this ratio was less than 2. Claus (1947), on the other hand, investigating β -amylase activity at pH 5.5 and various temperatures, with 20% maize starch paste as substrate, found reducing-group production (ferricyanide-ceric sulphate titration) to be a zero-order reaction up to 18% conversion. Freeman & Hopkins (1936), at 25°, pH 4.6, and with 2% (w/v) soluble starch solution, found that with low concentrations of the two enzymes saccharification (iodometric titration) was additive, though with higher concentrations of β -amylase the observed saccharification might exceed that calculated from the two component rates.

Although there is thus some agreement that an additive reaction is, in general, concerned—at least when the total hydrolysis achieved is not great—the data quoted do not afford a precise guide to conditions operating in the widely used standard methods for diastatic power determination of the Institute of Brewing (1933) and of the American Society of Brewing Chemists (1944) and in the proposed modification for determination of the individual enzymes. Accordingly, the problem has been reinvestigated using 2% and 1% (w/v) soluble starch substrates at 21° and pH 4.6, as a necessary preliminary to a more extended study of the joint action of the two enzymes.

EXPERIMENTAL

Reducing-group determination. In a modification of the Hagedorn-Jensen method, ferricyanide-carbonate reagent was used under conditions similar to those described by Lampitt, Fuller, Goldenberg & Green (1947), but with the range increased to determine a maximum of 18 mg. of reducing groups, calculated as anhydrous maltose. Thus, the ferricyanide reagent contained 33.0 g. $K_3Fe(CN)_6$ /500 ml., and the carbonate 42.4 g. anhydrous Na_2CO_3 /500 ml. For titration, 0.025 N- $Na_2S_2O_3$ was used, and the other reagents and the general conditions of working were as described by Lampitt *et al.* (1947). Standardized against a sample of maltose of known purity, under conditions of salt concentration identical with those encountered in the starch conversions, a linear relationship holds between maltose concentration and thiosulphate titration figures; 1 ml. 0.01 N-thiosulphate is equivalent to 0.368 mg. of maltose, a figure differing materially from that of Lampitt *et al.* (1947), but agreeing with that of the American Society of Brewing Chemists' method (1944), in which the relative proportions of ferricyanide, carbonate, and reducing groups during the reaction are substantially the same as those now used.

β -Amylase preparation. In a modification of the method of Hopkins, Murray & Lockwood (1946), ground barley was extracted with 2.5 times its weight of 20% (v/v) ethanol for 30 min. with stirring, the mixture filtered and the concentration of ethanol adjusted to 50% (v/v). After allowing to stand for 18 hr. the precipitate was

filtered off and discarded, and the concentration of ethanol in the filtrate adjusted to 80% (v/v). The precipitate, taken to dryness in the usual way and tested by the method of Preece (1947), proved to contain not more α -amylase than would produce 1/750 of the saccharification due to β -amylase (Table 1).

Table 1. *Effect of maintaining solutions of α - and β -amylases at 70° in presence of Ca^{++}*

(Samples of the enzyme solutions were allowed to act on 1% (w/v) soluble starch solution at 21° and pH 4.6 for 30 min.)

Time of maintenance at 70° (min.)	Reducing-group production (calculated as mg. maltose)	
	α -Amylase	β -Amylase
0	295.8	296.7
15	280.7	0.4
30	258.1	0.4
45	237.6	0.4

α -Amylase preparation. For this preparation a barley malt was used which had been specially dried at a temperature not exceeding 45°. The ground malt was extracted for 3 hr. with 20% (v/v) ethanol, and the filtrate adjusted to an ethanol concentration of 60% (v/v). The separated precipitate was taken to dryness to remove ethanol, redissolved in water and, after adding 0.2 g. calcium acetate/100 ml., maintained at 70° for 15 min. After cooling and removal of coagulated material, the α -amylase was reprecipitated and taken to dryness. β -Amylase activity could not be detected in such preparations (Table 1).

Soluble starch substrate. The sample of soluble starch used contained 0.5% of reducing groups (as maltose); solutions were prepared containing (a) 1.111 g. (dry substance)/100 ml., or (b) 2.222 g./100 ml., acetate buffer being added in each case to give a pH value of 4.6. Solutions were adjusted before use to 21°.

Starch conversions. Conversions of both 1 and 2% (w/v) starch solutions were carried out, 90 ml. of the bulk solution being used in each case. The unit of enzyme activity chosen was that amount of enzyme preparation which would produce in 15 min. reducing groups equivalent to 12.5 mg. of maltose from 1 g. of soluble starch in a total vol. of 100 ml. at pH 4.6 and 21°. The weights of the initial α - and β -amylase preparations containing 1 unit of activity were 0.8 and 0.2 mg. respectively. Later preparations were somewhat more active. The unit concentrations are symbolized as 1 α and 1 β respectively, and the approximate relative concentrations used are shown in Tables 2 and 3. Samples of solutions containing the appropriate amounts of enzyme, previously adjusted to 21°, were added to 90 ml. portions of the starch solution, and the total volume in each case adjusted to 100 ml. At appropriate intervals, samples of the conversions were pipetted into sufficient aqueous NaOH to give a total volume of 10 ml. at pH 10.5–11; reducing-group determinations were then carried out.

RESULTS

Results in Tables 2 and 3, which are corrected for reduction due to the starch itself and to the enzyme solution, are expressed in terms of theoretical

Table 2. *Liberation of reducing groups in 1% (w/v) soluble starch solution by varying proportions of α - and/or β -amylase*

(Temp., 21°; pH, 4.6; results expressed as percentage of theoretical maltose.)

Approximate relative concentration of enzymes	Reaction time (min.)					
	15	30	45	60	120	180
1 α	1.2	2.4	3.3	4.3	8.1	11.4
2 α	2.2	4.2	6.1	7.8	13.8	19.5
3 α	3.7	6.8	10.2	13.6	21.1	29.0
4 α	4.9	9.5	13.8	18.0	30.1	34.2
5 α	6.0	11.7	16.5	19.1	31.2	36.2
1 β	1.3	2.5	3.6	4.7	8.8	12.4
2 β	2.6	5.2	7.8	10.4	18.8	25.3
3 β	4.2	8.1	11.9	15.5	26.4	31.4
4 β	5.6	10.8	15.6	20.3	32.7	39.6
5 β	7.0	13.8	19.3	24.2	36.2	41.6
1 α + 4 β : Obs.	6.8	12.1	19.1	24.7	43.7	55.1
Calc.	6.8	13.2	18.9	24.6	40.8	51.0
2 α + 3 β : Obs.	6.3	12.4	18.0	22.4	39.6	51.2
Calc.	6.4	12.3	18.0	22.3	40.2	50.9
3 α + 2 β : Obs.	6.3	12.8	18.4	24.0	40.0	50.0
Calc.	6.3	12.0	18.0	24.0	39.9	54.3
4 α + 1 β : Obs.	6.2	13.1	18.0	22.8	37.6	45.4
Calc.	6.2	12.0	17.4	22.7	38.9	46.6

Obs. = observed; Calc. = calculated.

Table 3. *Liberation of reducing groups in 2% (w/v) soluble starch solution by varying proportions of α - and/or β -amylase*

(Temp., 21°; pH, 4.6; results expressed as percentage of theoretical maltose.)

Approximate relative concentration of enzymes	Reaction time (min.)					
	15	30	45	60	120	180
1 α	0.7	1.3	1.9	2.4	4.5	6.2
2 α	1.3	2.7	4.0	5.4	9.8	14.3
3 α	2.1	4.0	5.8	7.3	12.8	19.0
4 α	2.6	4.7	6.9	9.5	18.1	24.7
5 α	3.2	6.5	9.1	11.7	22.4	29.2
1 β	0.8	1.5	2.2	2.9	5.8	8.5
2 β	1.6	3.2	4.6	6.2	11.6	16.5
3 β	2.4	4.9	7.4	9.5	17.3	24.2
4 β	3.0	5.9	8.7	11.4	20.1	26.8
5 β	4.2	8.1	11.7	15.2	27.6	36.2
1 α + 4 β : Obs.	3.5	7.2	10.4	13.0	24.4	31.8
Calc.	3.7	7.2	10.6	13.8	24.6	33.0
2 α + 3 β : Obs.	3.7	7.7	10.8	13.5	26.6	34.4
Calc.	3.7	7.6	11.4	14.9	27.1	38.5
3 α + 2 β : Obs.	3.8	7.4	10.4	12.7	25.1	31.5
Calc.	3.7	7.2	10.4	13.5	24.4	35.5
4 α + 1 β : Obs.	3.2	6.5	9.7	12.6	22.7	30.0
Calc.	3.4	6.2	9.1	12.4	23.9	33.2

Obs. = observed; Calc. = calculated.

maltose, i.e. reducing groups calculated as maltose are returned as a percentage of the maltose theoretically obtainable by complete hydrolysis of the starch, it being assumed that 1 g. of starch would yield 1.055 g. of maltose. Direct comparison is possible of pairs of results (observed and calculated) bracketed together at the foot of each table, as the three determinations involved were performed simultaneously and using the same preparations of enzyme solutions. Direct comparison between

results with other proportionate enzyme concentrations is less exact, as this involves comparison of enzyme solutions necessarily prepared on different occasions.

DISCUSSION

Examination of the results given in Tables 2 and 3 indicates that, under the conditions used, a linear relationship between reducing-group production and time of reaction persists for β -amylase up to the

production of approximately 15% of theoretical maltose. This accords closely with the observation of Claus (1947), but is substantially lower than the limit of 30% given by Kneen & Sandstedt (1941), for linearity between reducing-group production and enzyme concentration; for the latter relationship, however, the present results give linearity up to approximately 25% suggesting, during the hydrolysis, a falling off in enzyme activity proportional to enzyme concentration. Hence, comparison of two β -amylase solutions giving conversions in the range 15–25%, while allowing correct relative results, will tend to give absolute results which are somewhat lower than the true values.

With α -amylase the relationships are less simple. Thus it may be agreed that true linearity with time does not occur. However, so long as the reaction time does not exceed 30–45 min., the departure from linearity up to 10% conversion is so small that very useful comparative measurements can be made in this range; in practice, little error is incurred if linearity is assumed up to 15%. With α -, as with β -amylase, proportionality between enzyme concentration and reducing-group production holds with considerable accuracy up to 25% hydrolysis. Thus, for comparison of the activities of two α -amylase solutions, conversions up to 25% may be employed, though for individual measurements for routine purposes a limit of 10–15% should not be exceeded.

For joint action, linearity up to 40% hydrolysis is usually assumed (Kjeldahl's law), but this assumption is not justified with the present methods. For the present results, proportionality between reducing-group production and time ceases in the range 20–25% hydrolysis for the whole series of β : α ratios employed and for both 1% (w/v) and 2% (w/v) soluble starch substrate. On the other hand, the additive relation in joint action can only thus be stated in simple terms: reducing-group production by α - and β -amylases acting together is truly additive during the linear phase of joint action. When linearity is departed from, an additive relation may persist, depending *inter alia* on substrate concentration and the β : α ratio. Thus, with 1% (w/v) soluble starch, a β : α ratio of 4:1 gives additive hydrolysis up to 25% theoretical maltose production; thereafter in the range studied the observed hydrolysis exceeds that calculated as the sum of individual actions. With α - and β -amylase concentrations of similar reducing group-producing potentialities, the joint action is still additive at 40–50% hydrolysis. With a β : α ratio of 1:4, there is a tendency for the observed hydrolysis beyond 25% to be somewhat below that calculated, a tendency which is seen with all the investigated ratios using 2% (w/v) soluble starch substrate.

Therefore, if the described ferricyanide technique is used for amylase determination, hydrolyses in

a period preferably not greater than 30 min. and not exceeding 15, 10–15 and 20–25%, production of theoretical maltose should be employed for β -, α -, and joint action respectively. Applied to brewery malt extracts, in which the β : α ratio is usually approx. 4:1 (Preece, 1947), determination of α -amylase activity and joint action (diastatic power) within the prescribed limits allows β -amylase activity to be obtained by difference. It would appear that, under the conditions described, α - and β -amylases function quite independently of one another.

It has recently been claimed by Blom & Røsted (1947) that the use of the ferricyanide method for determining reducing groups in solutions containing starch-conversion products tends to give high results as compared with copper-reduction methods. Thus, a solution which gave 42.0% theoretical maltose by ferricyanide reduction gave 34.9% with copper tartrate reagent (Fehling's solution); the surprisingly large discrepancy is probably due to a partial oxidative rupture of glucosidic bonds in oligosaccharides and polysaccharides. In view of the increasingly widespread use of modifications of the Hagedorn-Jensen method, because of its simplicity of operation and the high reproducibility of results, it is clearly important to know whether the suggested oxidative disruption of glucosidic linkages militates against obtaining accurate measurements. However, the method has been used with success by the American Society of Brewing Chemists (1944), results of diastatic power determination being returned as 'maltose equivalent' or, after dividing this figure by 4, as 'degrees Lintner'; the same divisor is employed when Fehling's solution is used for reducing-group determination, and it must be remembered that the definition of 'degrees Lintner' depends fundamentally on the use of Fehling's solution. Whether a figure somewhat greater than 4 should be taken as divisor following the use of ferricyanide is at present under investigation, but apart from this possible objection there is little doubt of the superiority of the ferricyanide method for this type of work; the end point is sharper than with Fehling's solution (especially for α -amylase conversions), and no difficulties arise in carrying out enzyme and substrate blanks.

SUMMARY

1. Using 1% (w/v) or 2% (w/v) soluble starch as substrate at pH 4.6 and 21°, reducing-group production (assessed by ferricyanide titration) by β -amylase shows a linear relationship with time up to 15% hydrolysis, and by the joint action of β - and α -amylase a similar relationship up to 20–25% hydrolysis. With α -amylase alone true linearity is lacking, but departure from linearity up to 10–15% hydrolysis is slight.

2. With $\beta : \alpha$ ratios varying from 4:1 to 1:4, the action of the two enzymes is truly additive up to 20–25% hydrolysis; beyond this point it may be equal to, greater than or less than the sum of

individual actions, depending on substrate conditions.

3. The significance of these facts in the assessment of amylolytic activity is discussed.

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Changes in the Extracellular and Intracellular Fluid Phases of Muscle During Starvation and Dehydration in Adult Rats

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It has been shown in a previous paper (Dicker, 1948) that rats fed on a protein-deficient diet develop tissue oedema very rapidly, i.e. the extracellular fluid phase of muscle and liver, as estimated in terms of chloride space, increases. The oedema starts at a time when the plasma protein concentration and the plasma colloid osmotic pressure are still normal. It seemed, therefore, of interest to investigate changes in the extracellular fluid phase of muscle in rats undergoing inanition or dehydration over short periods in which the plasma protein concentration was not decreased.

METHODS

Experimental animals. Adult male and female albino rats were used, of body wt. 250–320 g.

Diet. Some weeks before the experiment the animals were fed on a commercially prepared diet containing wheat offal 17.7, ground barley 8.8, white-fish meal 4.5, meat and bone meal 8.8, dried skimmed milk 14.0, dried yeast 1.2, salt 0.4, and cod-liver oil 0.4%. The total N content of the diet amounted to 3.26%, and its water content to 10.5%. The mineral content by analysis was 0.344 g. Cl, 0.210 g. Na, 4.100 g. K/100 g. and the calorific value 306.0 cal./100 g. During the period of experimentation the same type of food, but completely dehydrated, was given to one series of rats.

Analytical procedures. The following data were determined in each rat: (a) the content of water, chloride, sodium, potassium and nitrogen of heparinized plasma (in some cases, the urea concentration of plasma was also

estimated); (b) the content of water, chloride, sodium and potassium of muscle samples (the muscle used was the rectus abdominis); (c) the concentration of Cl^- , Na, K and N in the urine; urea and NH_3 in the urine were estimated in some cases.

Water content, Cl^- , Na and K concentrations were estimated in tissue and plasma samples, in the same manner and by the same methods as described in a previous communication (Dicker, 1948). Urea in plasma was estimated colorimetrically according to the method of Lee & Widdowson (1937), and the N content of plasma was determined by a micro-Kjeldahl method.

In the urine, Cl^- was estimated according to Volhard (1878), and Na and K by the method of McCance & Shipp (1933). NH_3 was adsorbed on permutit and estimated after nesslerization (Folin & Bell, 1917), and the urea determination followed the method of Scott (1940). The specific gravity of urine was determined in the apparatus of Heller (1940), using a mixture of carbon tetrachloride and light petroleum. All data for muscle are expressed per 100 g. fat-free tissue (see Hastings & Eichelberger, 1937).

Estimation of the extracellular and intracellular fluid phases of muscle. The extracellular and intracellular fluid phases of muscle were calculated on the assumption that all the Cl^- is extracellular, and that its concentration is that of an ultrafiltrate of serum (Fulton, 1947). The volume of the extracellular fluid phase was determined by calculation (Hastings & Eichelberger, 1937).

The amount of potassium in the intracellular fluid phase was calculated as follows:

$$\text{m-equiv. intracellular K/kg. muscle} = (\text{K})_M - (\text{H}_2\text{O})_F \times (\text{K})_F,$$

where $(\text{K})_M$ = m-equiv. K/kg. muscle, $(\text{K})_F$ = m-equiv.