CHAPTER IV -

- DISCUSSION
Glucoamylase is formed by fungi, mammals, a few bacteria such as Clostridia (Hockenfull and Herbert, 1945) (French and Knapp, 1950) and perhaps plants also (Takahashi et al., 1971). The mammals do not seem to form β-amylase and hence they must have a mechanism of producing maltose/glucose from dextrin. This is provided by the glucoamylase (Rosenfeld et al., 1968; Seetharaman et al., 1970). The fungi, however, seem to possess α-, β- and glucoamylases as well as many α- and β-glucosidases (Windish and Mhatre, 1965; Greenwood and Milne, 1968; Takagi et al., 1971). Among the fungi, the general Aspergillus, Rhizopus and Endomyces seem to be rich sources of glucoamylase (Hattori, Starke, 1965); 2 per cent starch - 0.5 per cent yeast extract media was used for screening purposes. The medium has been used for a similar purpose in the case of yeasts. In our screening experiments for the glucoamylase-producing strains, it is the Aspergillus, and more particularly the strains A. niger NRRL 330 and 337 which were found to be the best ones among the strains tested. A. niger enzymes are reported to contain glucoamylase with a high temperature optimum of 60°C and a comparatively low pH optimum of 4; both these factors are advantageous for avoiding contamination by bacteria. The strains apparently vary widely in their ability to form glucoamylase. Underkofler (1969) reported that A. niger NRRL 330 produced 84 units/ml in a medium containing
The strain of *A. niger* NRRL 350 used here for nutritional and biochemical studies has also presumably been used by Pazur (1962), Lineback (1972) and Underkofler (1969) for nutritional, biochemical and industrial studies. The nutritional experiments mentioned here were conducted some years ago at about the same time as those of Lineback (1966). However, there are differences in results. Similarly there are marked differences in biochemical characteristics of the glucoamylases also. We are not certain what these differences are due to and whether our strain has undergone any genetic changes. Another point to be observed is that many workers including Pazur and Lineback have used Diazyme as the source of glucoamylase for biochemical studies and assumed that it (Diazyme) is from *A. niger*. Underkofler describes the development of this process of fermentative production of glucoamylase (1969); the strain selection, pilot plant trials etc. have all been conducted with reference to *A. foetidus* and a mutant derived from it. Diazyme is derived from a mutant of *A. foetidus* and not from *A. niger* NRRL 350 (Underkofler, personal communication, 1973). However, a recent publication by Pazur has specifically employed *A. niger* NRRL 350 (Pazur, 1972).

The glucoamylase production by the fungus seems to proceed at a basal rate during the active growth of the fungus (first 48 hrs) but rises sharply after the growth is almost complete. Perhaps the formation
is a response induced by the substrate-inducer. During the growth phase, the amount of enzyme formed is probably the maximum commensurate with growth rate and is limited by the competing synthesis of proteins for other cellular activities including growth. Once the active growth and multiplication cease, the formation of glucoamylase sharply rises provided the inducer, in this case starch/dextrin/maltose, is still present.

\( \text{A. oryzae} \) has also been reported to produce glucoamylase (Morita et al., 1966). \( \text{A. foetidus} \) and its mutant produced as much as 176 and 288 units/ml, \( \text{A. phoenicis ATCC 13157} \) and \( \text{A. niger NRRL 337} \) were comparatively poorer (93 and 65 units/ml) producers of glucoamylase respectively (Underkofler, 1969). Thus it is seen that even the best yield of glucoamylase obtained here (22 units/ml in the 14 per cent jowar medium) falls far short of what has been reported (84 units/ml) by Underkofler (1969) for the same strains. It is not clear whether this discrepancy is due to differences in growth conditions or to a "degeneration" of the strain \( \text{A. niger NRRL 330} \). But it is certain that mutation to higher production of any metabolite can be achieved.

It has been well recognized for long that many factors influence the quantity of amylase formed. It was recognized by Wortman (1882) for many fungi, Saito (1911) for \( \text{A. niger} \) and \( \text{A. oryzae} \) that amylase was formed only when the growth medium contained starch; whether or not maltose had any effect is not clear. However, more recently, it has been
reported (Raghavendra Rao and Sreenivasaya, 1947) that starch and maltose promoted the formation of "\[\alpha\]-amylase" by A. oryzae; glucose also had a pronounced effect although only 50 per cent as effective, but Tomomura et al. (1953) have reported that glucose caused inhibition of amylase formation in A. oryzae. In the present studies on A. niger NRRL 350, only maltose and starch were found to be effective. Most other sugars tried not only did not promote either enzyme production or good growth. Apparently, the quantity of enzyme formed increases with increased content of starch in the medium up to a limit; there is no significant difference in enzyme formation in media containing 6 per cent and 10 per cent starch. Higher concentrations of starch depress glucoamylase production in media not containing organic nitrogen compounds.

The effect of the source of nitrogen on glucoamylase formation is quite noticeable. Complex N-sources such as yeast extract, peptone and corn steep liquor, promoted good growth and enzyme formation. But other complex materials (extracts of oilseed cakes, casein or its hydrolysate) were not as beneficial. The inorganic sources of nitrogen were definitely much poorer in promoting growth and enzyme formation: urea and ammonium sulphate were better than others. Using the same strain A. niger NRRL 350, Lineback (1966) reports that NaNO₃ was a poor source but NH₄Cl was a fairly good one. Those nitrogenous sources containing anions which could either be utilised by the organism (acetate, nitrate, sulfate, etc.)
or serve as a buffer (phosphate) could serve as a better source of nitrogen. A similar finding was reported by Raghavendra Rao and Sreenivasaya (1946) who found that ammonium acetate and sodium or potassium nitrate were better sources of nitrogen than ammonium sulphate or other inorganic ammonium salts. Lineback (1966) has also observed that organic nitrogen sources such as peptone or yeast extract were superior to inorganic nitrogen sources for glucoamylase production: at higher levels of carbohydrates and organic nitrogen, there was better formation of enzyme. It is clear that the complex organic nitrogen source supplied compounds other than mere amino acids, vitamins and trace metals since casein hydrolysate, vitamin trace metal mixtures together are not as effective.

The mineral constituents do not seem to exert much effect on glucoamylase formation at 6 per cent starch concentration. Although corn starch AnalaR (BDH) was used, it might contain enough minerals for growth and enzyme formation. Deficiency of phosphate in the medium has been reported to result in a film adsorption of amylase but not glucoamylase by the mycelia of A. oryzae (Tonomura et al., 1963). In the case of A. niger, a similar phenomenon is noticed at lower pH values (Lineback et al., 1966). In the present instance, glucoamylase was found to be present to the same extent in phosphate-"dependent" and "sufficient" media.
The presence or absence of trace metals do not seem to make any difference for glucoamylase formation in these experiments. Lineback (1966) reports that non-addition of trace metals depresses the amount of glucoamylase formed. The lack of effect of trace metals observed here is perhaps due to the quality of the chemicals and starch which may contain enough trace metals.

Supplementation of the medium with vitamins also had no noticeable effect on glucoamylase production. Since the organism can synthesize the needed vitamins, the results are not altogether unexpected.

The proportion of carbohydrate to peptone or the nitrogen source seems to have significant effect on growth as well as glucoamylase production; a ratio of 20:1 seems to be best. Whether other related enzymes (α- or β-amylase transglucosylase, etc.) are also affected has not been tested. However, the pH in most cases decreases to 3 to 2.2 and therefore it is unlikely that these enzymes will survive.

Comparing the various nutrients tested, it becomes evident that complex nitrogen sources such as peptone are not equivalent to a complex food material such as jowar (at equivalent starch and nitrogen levels). In 10 per cent jowar media, 22 units/ml of glucoamylase are produced whereas only about 10 to 14 units/ml are produced in 3 per cent starch, 0.5 to 1 per cent peptone or yeast extract. Growth in a chemically
defined medium (Table 19) leads to the formation of almost as much glucoamylase as in medium containing 6 per cent starch and 0.5 per cent yeast extract. These results would indicate that natural food materials such as jowar (Sorghum vulgare), ragi (Eleucrium coracana), corn (Zea mays) contain as yet unrecognized compounds which cause increased production of glucoamylase (Table 4). The results show that jowar and ragi were the best substrates for glucoamylase formation. But the amount of enzyme formed by the same strain A. niger NRRL 350 in 15 per cent corn - 2 per cent corn steep liquor as reported by Underkofler (1969) is 4 times higher than found here.

Growth and quantity of glucoamylase formed do not seem to go hand in hand. Presumably most of the growth (cell multiplication) would be complete in 48 to 72 hours in shake flasks whereas an equal amount or the bulk of enzyme seems to be formed after this period (Tables 2 and 3). Similar instances in other fermentations may be recalled; citric acid fermentation by A. niger (Steel et al., 1954), bacterial amylase production by B. subtilis (Fukumoto et al., 1957) etc. The substrates presumably present continue to act as inducers; only a minor part of the initially added inducer would have been utilised at the end of the growth phase. However, the maximum yield of enzyme in shake flasks seems to be on the 5th day after which there is hardly any increase. In fermentor where aeration and agitation are expected to be better, the maximal yields are reached only after 96 hours even when a
10 per cent of 24 hr grown inoculum is used. Underkofler (1969) reports that in 64 hours, the enzyme yields reached high levels (450 units/ml); with double the aeration rate, the maximum yields were obtained within 64 hours. The growth rate and indirectly enzyme formation are related to the aeration-agitation of the media.

The pH of the medium does not affect significantly enzyme formation over a wide range: an initial pH of 4 to 4.5 seems beneficial. Although in most cases of good glucoamylase yield, the final pH was around 2, keeping the pH constant at 4 did not seem to affect enzyme yield. In plant yields a final pH of 3.7 to 3.5 has been reported by Underkofler (1969). Lineback (1966) showed that glucoamylase is not adsorbed on to cells at pH 4 or below as happens for α-amylase (Tokumura et al., 1963; Lineback et al., 1966). No α-amylase was detected in the medium. This may be due either to adsorption of the enzyme on to cells at the low pH prevailing at the time of harvest, to its destruction at the pH or to a preponderance of glucoamylase in the medium. The results obtained do not clarify this. Lineback (1966) and Kerr (1961) observed that formation of amylase is inhibited at pH 4.2 and that glucoamylase is not inactivated in the pH range 2 to 9.

Temperature of growth between 28° and 37° did not affect significantly glucoamylase yields in 120 hours. Underkofler (1969), however, reports that in 40 litre-fermenters, increase of temperature
from 32° to 36.6° resulted in a fall in glucoamylase activity from 510 units/ml to 400 units/ml. The fungal glucoamylase preparations generally contain transglucosylase also. Different methods are used to remove transglucosylase because its presence is not desirable in glucose production. But, in the present instance, transglucosylase could not be detected in the culture filtrate, concentrates, or the purified enzymes. Whether this is due to culture conditions, as Lineback (1966) has found, is not clear.

Effect of aeration on glucoamylase formation is striking. Stationary culture is quite unsatisfactory. But it was somewhat unexpected to find that reciprocating motion also did not prove efficient. Lineback (1966) has used this type of shaking and reports good yields. Since he does not report enzyme yields, the results cannot be compared. Growth on rotary shaker gave good results. A volume of 50 to 90 ml of media per 250 ml Erlenmeyer flasks does not affect enzyme yields. This corresponds to a surface volume ratio of about 1.57 to 0.9 and oxygen transfer of 0.44 to 0.25 mmole/lit/min. Presumably as long as this much oxygen is available, growth and enzyme yields would be optimal. In industrial practice, apparently oxygen concentration and transfer in fluids are increased by keeping a head pressure of 15 psig, higher aeration rates and good agitation (Underkofler, 1969).
The type of inoculum used - spores, or 24 to 48 hour vegetative growth, percentage of vegetative inoculum, the period of storage prior to inoculum preparation - does not seem to affect enzyme yields (Tables 11 - 12). The industry seems to use a vegetative inoculum. The advantage presumably is the saving in agitation-aeration of huge bulk of thick mashes for 24 hours (since fermentation time is reduced about 24 hours by using a 24 hour-vegetative growth). The mycelial (pulpy) form of inoculum (24 hour-growth) seems to be better than a pellet type one, perhaps because oxygen transfer to cell surface is much better with mycelial forms. Ikahashi (1965) reports such a beneficial effect in citric acid fermentation. These are in contrast with the observations of Steel et al. (1954) in citric acid fermentation where a pellet form produces more citric acid.

Among the cereals tested, Jowar and ragi were better than the others. Corn and its products have been utilised as raw materials for glucoamylase production (Tsuchiya, 1949; Pan et al., 1953; Bailey, 1971; Indian Patent 115837, 1963). Jowar and ragi are cheaper compared to the other food grains in India and corn also may become cheaper and more available now. Fermentative trials with 14 percent jowar mash thinned by acid gave in 96 hours enzyme yields comparable to shake flask experiments. However, these yields (18 units/ml) are much less than those reported for this culture (64 units/ml), A. foetidus (175 units/ml) and its mutant (280 units/ml) (Underkofler, 1959). The enzyme produced
in the fermentor has been compared with some commercial preparations and with preparations from other locally available strains. It is comparable to the commercial ones in activity towards starch and in conversion of starch to glucose, but are likely to cost more than Diastase since the yields of latter per unit of the raw material are almost 10 times higher than reported here.

Purification

Glucoamylase from some sources has been purified to homogeneity: A. niger species (Smiley et al., 1971; Linesback et al., 1969; Pazur et al., 1966; Linesback et al., 1972; Fleming and Stone, 1965; Linesback and Bauman, 1970; Tsujisaka et al., 1958), A. awamori (Smiley et al., 1971), A. oryzae (Morita et al., 1966), rice (Takahashi et al., 1971). Most of the procedures used above include alcohol precipitation followed by ion exchange (DEAE-cellulose or CM-cellulose chromatography). In one instance, rice \( \alpha \)-glucosidase has been purified by fractionation with ammonium sulphate, CM and DEAE-cellulose fractionation, to obtain two homogeneous \( \alpha \)-glucosidases both of which act on starch and maltose to release glucose (Takahashi et al., 1971). In another case, glucoamylase of A. niger was fractionated by a preliminary treatment with lead acetate followed by isopropanol precipitation and fractionation on DEAE-cellulose; there seemed to be three separable fractions with glucoamylase activities (Fleming and Stone, 1965). But no details are available in this note. Another report mentions of the formation of two isoenzymes of
glucoamylase in one medium (containing dextrin with only 3 per cent reducing sugars) but only one in another (with dextrins containing 58 per cent reducing sugars (Kawamura and Sawai, 1968).

The fungus A. niger NRRL 330 was grown as described in previous sections in 20-litre fermentor and the culture filtrate was used as the source of the enzyme. Purification of the enzyme is comparatively simple and easy since only three steps - concentration, alcohol precipitation and DEAE-chromatography - are necessary to obtain homogeneous preparations: a 30-35 fold purification leads to homogeneous enzyme(s). The fractionation procedure is similar to the procedures described by Pazur (1959) and Lineback (1972). But instead of adsorption at pH 8 and a stepwise elution by buffers with decreasing pH, the adsorption of the enzyme was effected at pH 7 and elution also at pH 7 but with an increasing NaCl concentration. The results obtained are also different. Three separable enzyme activities were obtained instead of only two, as reported by Pazur (1959) and Lineback (1972). These results have been obtained at least 5 times. The major glucoamylase accounts for 63 per cent of the total activity, the second 31 per cent and the third 5.5 per cent; all of them produce glucose from starch and maltose. Hence these glucoamylases (at least one of them) seems to be different from the ones described in literature (Pazur, 1959; Lineback, 1972).
In all the above cases, the criteria for purity have been discussed. Gel electrophoresis at different pH values and ultracentrifugation. On the basis of these criteria, the purified glucoamylase from *A. niger* (i.e., DIAZYMES of Miles Lab.), *A. phoenicea*, *A. awamori*, *A. oryzae* and *Rhizopus delemar* are homogeneous. The ones reported in this thesis are pure by these criteria except that two of the fractions A and C have not been examined ultracentrifugally.

In most of the above cases, at least two isoenzymes of glucoamylase seem to be formed; they have been separated and studied well in some cases (e.g., Diazyme preparations). In an interesting paper, Kawamura and Sawai (1968) have shown that the two glucoamylases are formed by *Candida pelliculosa* when the yeast is grown in a medium containing starch or dextrin with little reducing power whereas only one isoenzyme is formed when grown on well-degraded starch. The growth media used in Diazyme production and in experiments conducted here employ whole grain which contain mostly starch and a little reducing sugar. If the observation on *Candida pelliculosa* is correct and applicable here, the formation of more than one isoenzyme can be expected.

**Temperature effect**

The three fractions seem to be similar in their temperature optima (50° - 60°) for activity. S.A. for A - 18.5; S.A. for B - 37.0 and S.A. for C - 7.2. And thus, they resemble the other glucoamylases
described in literature in their temperature optima.

The glucoamylase fractions, however, seem to differ from one another and from the Diazyme isolate (Lineback, 1969) in their heat stability. The latter is reported to be stable for 30 min at 60° with the activity decreasing rapidly beyond 60°. Some of these preparations are quite stable on storage of enzyme solution at 4° at pH 4 for as long as 6 years (Pazur, 1970) and a concentrated solution (77 mg protein/ml) of DIAZYM® (R) keeps for one year at least at room temperature (Underkofler, 1969). But, another story indicates that the enzyme from A. phoenicis loses 45 per cent activity after 192 hours at room temperature (Lineback, 1970). We have found the enzyme solutions of alcohol precipitate (S.A. 22.9) can be stored for at least a year at 4°. Among the three fractions, only the first fraction (S.A. = 19) shows some resistance to heating at 60° in the absence of substrate, retaining 65 per cent activity after 10 min at 60°; the others are rather heat-labile.

pH effect

The glucoamylases have a broad pH activity profile from 3 to 8 with the optimum around pH 4 to 4.5 (Lineback, 1969; Whittaker, 1972). The three fractions also conform to this pattern: each retains as much as 40 - 45 per cent activity at pH 1.5 and 8, with maximal activity at 4 to 4.5. The enzymes are quite stable at pH 2 at least
for 2 - 3 days at 30° (cf. Smiley et al., 1971) and for a year or longer at pH 4 at 4°.

Carbohydrate content

Like the rest of the glucoamylases, the three glucoamylases isolated here are also glycoproteins. The most abundant fraction resembles glucoamylase II of Pazur (1970) and Lineback (1972) in its carbohydrate content (16 per cent). The monosaccharides present have, however, not been determined in any of the fractions. The other two contain 8.2 and 6.2 per cent carbohydrates, less than what has been reported by others for glucoamylase I of Diazyme®. The first report on the carbohydrate content of glucoamylase I isolate from Diazyme® indicated a 10.7 per cent content (Pazur, 1963). Since then, different values have been reported: 15 per cent (Pazur, 1969) and 15 per cent Lineback, 1972). It is not possible to state whether this is due to the different proportions of carbohydrates present in different batches of the Diazyme or to a variation in carbohydrate estimation. The latter is unlikely. It is probable that the extent of incorporation of carbohydrate into the protein may vary with growth conditions; more carbohydrate being taken up under conditions of surfeit. It is known that Taka-Asylase A free from carbohydrates is produced when A. oryzae is grown in a medium free from carbohydrates (Hanafusa et al., 1955). The carbohydrate moiety does not appear to be essential for activity but quite important for the
stability of the enzymes (Pazur, 1970). Removal of 66 per cent of the carbohydrates by periodate treatment and dialysis abolished 50 per cent of the activity (Pazur, 1970). Results of periodate treatment here indicate that at 0.1 M concentration, periodate abolishes the activity in 10 min with the removal of 97 per cent carbohydrate. Even prolonged treatment (24 hours) with 0.02 M periodate results in partial loss of activity: a 40 – 50 per cent loss in activity and 50 to 70 per cent loss of carbohydrate. According to Lineback (1972), glucosylases I and II contain the same carbohydrates but have different amino acid composition. They contain predominantly mannose (8 and 12), glucose (2 and 3) and galactose (0.2 each) respectively (Pazur, 1962).

Glucosylases of A. phoenicis (Lineback, 1970) and of A. oryzae (Morita et al., 1966) seem to contain 17 per cent and 30 per cent carbohydrate respectively. There does not appear to be any reasonable explanation for the widely varying carbohydrate content of these enzymes.

Molecular weight: subunit structure

The molecular weights of the different fractions of glucosylases from various sources have been determined by a single or a combination of the following procedures: gel filtration, SDS-polyacrylamide electrophoresis, and ultracentrifugation. Pazur (1962) first reported that glucosylase I had a M.W. of 97,000 ± 5 per cent by ultracentrifugal
analysis and later density gradient ultracentrifugation (1970) gave a M.W. of 110,000. Lineback obtained molecular weight values of 74,900 ± 1500 for I and 54,300 ± 6690 for II by ultracentrifugation (Archibald) and 70,000 and 59,000 by gel filtration for glucoamylases I and II respectively derived from Diazyme (A. niger ?) The three fractions obtained here had molecular weights 90,000, 71,000 and 72,000 by gel filtration and 81,000, 69,000 and 70,600 by SDS-polyacrylamide gel electrophoresis respectively. Reasons for this difference (of 8000) in the molecular weight of fraction II as determined by the two methods is not clear. The different values for M.Ws of glucoamylase I (Diazyme) is to be noticed; 97,000 (Pazur, 1962) to 110,000 (Pazur, 1970) and 61,500 (Lineback quoted by S.A. Barker, B.B.R.I. 1971) to 74,900 (Lineback, 1972). Whether these differences are inherent in the errors or variations in determination is not clear.

The three different fractions of glucoamylase isolated here seem to be different from the glucoamylases I and II described by Pazur and by Lineback.

Other glucoamylases have molecular weights from 57,000 to 71,600: A. phoenicis has two isoenzymes. The molecular weight of one of them has been determined and found to be 63,600 (Lineback, 1970). One of the four glucoamylases of A. oryzae (Ohga et al., 1966) has a molecular weight of 69,000. A. awamori has two glucoamylases with
<table>
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<tr>
<th>Molecular weight (Dalton's)</th>
<th>Carbohydrate content %</th>
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<tr>
<td>Glucosamylase I</td>
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<tr>
<td>97,000 ± 5%</td>
<td>10.7^c</td>
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<td>110,000^b</td>
<td>15^d, 15^e</td>
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<td>61,500^g</td>
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<td>Glucosamylase II</td>
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<td>54,300^f</td>
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a  Pazur and Kleppe (1962) - Ultracentrifugation  
b  Pazur (1970) - Density gradient  
c  Pazur (1963)  
d  Pazur (1969)  
e  Lineback (1969)  
f  Lineback (1972) - Ultracentrifugation  
g  Barker (1971)
molecular weights 71,600 and 57,600 by gel filtration and 74,900 and 54,600 (presumably by ultracentrifugation) (Smiley et al., 1971).

The molecular weights of different glucoamylases lead one to suspect that these molecules may consist of subunits. Lineback (1969) found ultracentrifugally that the glucoamylases isolated from DIAZYME do not dissociate in 8 M urea at pH 4 and 2 into smaller units. The other properties were not tested. The experiments conducted here showed that the presence of 8 M urea in the reaction mixture did not effect the activity of the enzyme. In contrast, guanidine hydrochloride at higher concentrations (2 M) abolishes the activity completely and most of the activity could be recovered by overnight dialysis, i.e., removal of guanidine hydrochlorides. These results and also those of Lineback (1969) would indicate that the glucoamylases (at least those which have been tested) do not have a subunit structure.

**Effect of photooxidation and some group-specific reagents**

Some attempts have been made to study the active sites of the glucoamylases by using inhibitors, group specific agents and photooxidation. Photooxidation of glucoamylase B (of *A. niger*) at pH 5.5 did not effect the activity nor were tryptophane, cysteine or tyrosine significantly destroyed. On the other hand, there was marked loss in activity at pH 9 and also in tryptophane content. A low level (0.01 per cent) of methylene blue was used in the above experiments;
methylene blue is also reported to be rather non-specific (Wested, 1965). Using a higher concentration (0.01 per cent) of Rose Bengal or methylene blue, there was no effect on activity at pH 4, but at pH 8.9, there was 40 to 50 per cent inactivation in 30 min; the histidine content of the irradiated protein was not determined. Since Rose Bengal is presumably specific for histidine (Wested, 1965), one would conclude that histidine residues are either part of the active site or essential for maintaining the three-dimensional structure of the enzyme molecule.

The group specific agents tested were not found to affect activity (Table 38). Diisopropyl phosphate which blocks the active serine in serine-proteases (Jensen, 1949) had no effect even at 0.02 M. The SH-blocking reagents, iodoacetamide, p-chloromercuribenzoate and NEM did not affect activity. NBS is assumed to be a drastic reagent which affects many amino acid residues but even this was ineffective. EDTA also did not reduce the enzyme activity. The absence of any observable effect of any of these reagents would lead to the conclusion that Ca++ (or divalent cations), serine, -SH groups (cysteine), tryptophane-histidine-tyrosine are not part of the active site, nor are they required for the integrity of the three-dimensional structure of the enzymes. However, this conclusion should be regarded as purely tentative, since only one set of experimental conditions was tested and since other possibly more effective conditions have not been tried.
N- and C-terminal amino acids

The only report of the determination of the N-terminal amino acid(s) is that of Lineback (1969) who found that both glucoamylases I and II had alanine at the N-terminal. The amino acid analysis of the two enzymes has revealed the presence of 9 to 12 lysine residues in these enzymes (Lineback, 1972), but during the preparation of the DNP-derivatives, no DNP-derivative was obtained in the aqueous phase (Lineback, 1969). But, we have obtained a water soluble DNP-derivative of lysine. In the present instance, however, all the three fractions had phenylalanine as the N-terminal amino acid and the C-terminal one, leucine. Thus, these three fractions are set apart from those of DIAZYME by yet another criterion.