Studies on Carbohydrate-Metabolizing Enzymes

12. A SURVEY OF THE CARBOHYDRASES OF ALFALFA*

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The presence of a number of carbohydrases in alfalfa (lucerne, *Medicago sativa*) seeds and seedlings has been reported, including α-D-glucosidase, α-D-galactosidase, β-D-fucosidase, β-*N*-acetyl-D-glucosaminidase (Levvy & McAllan, 1962), β-D-glucosidase, β-D-galactosidase and α-D-mannosidase (Hill, 1934; Levvy & McAllan, 1962). A comparative study of almond and alfalfa 'emulsins' has also been made (Hill, 1934). Studies of the mature plant appear to be limited to the pectinesterase activity (Lineweaver & Ballou, 1945; MacDonnell, Jansen & Lineweaver, 1945; MacDonnell, Jang & Jansen, 1950). In most previous studies, glycosidase activities were tested against synthetic and natural glycosides with non-carbohydrate aglycones. A survey of the oligosaccharide- and polysaccharide-degrading enzymes has now been undertaken with a view to the assessment of their possible use in the structural analysis of alfalfa components.

The carbohydrases probably bear some relation to the carbohydrate components of the plant. Apart from the reserve galactomannan of the seed (Hirst, Jones & Walder, 1947; Courtois, Anagnostopoulos & Petek, 1958), and the pectic substances and hemicellulose of the mature plant (Aspinall & Fanshawe, 1961; Myhre & Smith, 1962), little is known of the high-molecular-weight components of alfalfa. Small quantities of starch are present in the leaves of the plant (Hirst, Mackenzie & Wylam, 1959), though this is likely to show a marked diurnal variation, as in the related clover (Bailey, 1958). Fructosans, which form the main carbohydrate reserves of grasses, appear to be absent (Hirst *et al.* 1959). The raffinose–stachyose series of oligosaccharides, together with sucrose, appear to comprise the oligosaccharide component in alfalfa (Aspinall & Fanshawe, 1961; Meunie, 1936).

EXPERIMENTAL

Materials

Plant material. Medicago sativa var. de Puits was used.

Seeds. These were purchased from Elsom Ltd., Spalding, Lincs., and were untreated.

Seedlings. This term refers to seeds that had been steeped overnight in water and then stored, moist, in a layer 0·5 cm. thick between four sheets of Whatman no. 3 filter paper for 4 days, at 26°, in the dark. For the preparation of seedling extract S, the seeds were steeped in water containing benzyl penicillin (6 mg./l.), streptomycin sulphate (10 mg./l.) and Mycostatin suspension (2 ml./l.) (E. R. Squibb and Sons, Speke, Liverpool), and the sheets of paper were also moistened daily with this solution.

Leaf and stem tissue. Alfalfa was cut on 24 October 1962 (third crop) before flowering, separated into leaves and stems and deep-frozen (−20°) within 3 hr. of cutting.

Substrates. Nigerose was prepared by the acetolysis of dextran as described by Matsuda, Watanabe & Aso (1961). Isolichenin was isolated from Iceland moss (Peat, Whelan, Turvey & Morgan, 1961). Galactomannan was isolated from alfalfa seed according to the method of Courtois *et al.* (1958). Nigeran was a gift from Dr H. Weigel (Royal Holloway College). Soluble nigeran was prepared by heating a mixture of nigeran (1%) in N-H_2SO_4 for 20 min. at 100°, cooling, neutralizing, dialysing and freeze-drying. The remaining carbohydrates were laboratory or commercial samples, whose purity had been checked by paper chromatography.

Methods

Paper chromatography. Chromatograms were developed with solvent *A* [ethyl acetate–pyridine–water (10:4:3, by vol.)], or solvent *B* [ethyl acetate–acetic acid–formic acid–water (18:3:1:4, by vol.)] when acidic sugars were expected as products. Sugars were located with a silver nitrate–sodium hydroxide reagent (Trevelyan, Procter & Harrison, 1950) and *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950).

Quantitative measurements. Nitrogen content of extracts was measured by a semi-micro-Kjeldahl procedure (Chibnall, Rees & Williams, 1943). Soluble protein was measured with a colorimetric biuret reagent (Robinson & Hogden, 1940). Reducing power was determined with a 3,5-dinitrosalicylic acid reagent (Sumner, 1924), and glucose with a specific glucose oxidase reagent (White & Subers, 1961).

α-Amylase assay. This was carried out by measurement of the decrease in iodine staining power of British Drug Houses Ltd. soluble starch (cf. Van Dyk & Caldwell, 1956).

pH *optima of enzymes.* Hydrolytic activities of a seedling enzyme preparation containing 52% of protein were determined on various substrates over the pH range 2–8 at intervals of about 1 pH unit. Digests (total volume 4 ml.) containing substrate (0·25%), 1 ml. of buffer (McIlvaine, 1921) and 1 ml. of dissolved enzyme preparation (1–4 mg./ml., depending on the activity) were incubated for 18–24 hr. at 37°. Enzyme activity was stopped by heating at 100° for

* Part 11: Cunningham & Manners (1964).

3 min. Parts of the solutions were appropriately diluted and the glucose content or reducing power of each was determined. Appropriate enzyme and substrate controls were prepared and analysed. The glucose oxidase reagent gave zero readings with all substrates.

RESULTS

Extraction of soluble protein from alfalfa tissue. A variety of methods for tissue disintegration were tested. The seeds were milled to a fine flour before extraction. Seedlings and leaves were equally well macerated with a Waring Blendor or a motor-driven pestle and mortar. Stems presented some difficulties, but these were overcome by the use of an Ultra-Turrax TP 18 homogenizer, after the stems had first been cut into 1 cm. lengths. This instrument was later used for all tissues. Batches (100 g.) of leaf, seedling and seed required 3 min. for efficient maceration, and stem required about 10 min.

Sodium acetate, pH 7·5 (0·4M), sodium citrate, pH 6·7 (0·2M), and potassium chloride, pH 6·0 (0·7M), were examined for efficiency in extracting enzyme protein from seedlings. (The detailed procedure is described below.) Protein extraction (as measured by the biuret method) was of the same order for the three solvents, and the laminarinase activities of the solutions were in the proportions 10:10:7. The rates of hydrolysis of melibiose and lactose were not affected by the method of extraction. Since extracts made with 0·4M-sodium acetate were most easily clarified on centrifugation, this solvent was chosen for most extractions.

Several extracts of each type of tissue have been prepared; a typical extraction is described below, although slight modifications were made in some experiments. Tissue (100 g.) in about 300 ml. of 0·4M-sodium acetate at 0° was macerated with cooling. The mixture was then stirred at 20° for 1 hr. After centrifuging (at 2000*g* for 20 min.), the pH was adjusted to 6 with acetic acid solution and the extract dialysed against running tap water (6°) for 20 hr. The solution was then centrifuged again and the supernatant solution (about 400 ml. at 0°) brought to 70 % saturation by the addition of solid ammonium sulphate. After 1–2 hr. at 0°, the precipitate was collected, dissolved in water, dialysed against water at 2° for 20 hr. and centrifuged again. The supernatant solution was made 0·01M with respect to sodium citrate buffer, pH 6·0, and stored as such at 2° or freeze-dried. For seedling extract S, 0·4M-sodium acetate containing the three antibiotics was used for maceration, and the extract dialysed in the presence of thymol.

A modification of the above procedure was necessary when extracting seed. If the first dialysis stage was prolonged for 2 days, most of the reserve galactomannan was precipitated and could be removed by centrifugation. This did not happen with the other tissues.

In small-scale experiments with dry seed, moist seedlings and deep-frozen stem and leaf (30 g. batches), the yields of protein with the above procedure were about 0·5, 0·5, 0·14 and 0·4 % respectively, though these values were often lower in larger-scale experiments. Control experiments with seedling and leaf showed that negligible protein (biuret estimation) or laminarinase activity was obtained on re-extracting the tissue. Extracted washed stem homogenate showed only weak maltase, amylase and laminaribiase activities and no α- or β-galactosidase or 'pectinase' activity when incubated with appropriate substrates. In test experiments, laminarinase and melibiase activities were not affected by dialysis against tap water. Freeze-drying without salt led to a variable loss of activity, but this was overcome by freeze-drying in 0·01M-sodium citrate solution.

Specificity of the carbohydrase activities. Digests were prepared containing 10 mg. of substrate in 1 ml. of sodium acetate buffer, pH 5·2, 1 ml. of enzyme preparation (protein concentrations: seedling, 0·5 %; stem, 0·05 %; leaf and seed, 0·15 %) and toluene. After incubation for about 20 hr. the solutions were analysed by paper chromatography. The results of visual estimation of the chromatograms are shown in Table 1. These values are averages of several experiments. In general, the seedling extracts show highest activity; this is partly a reflexion of their higher protein content (cf. stem) although their specific activities seem to be higher than those of seed extracts. There does not appear to be any marked variation in type of activity with source. The extracts thus contain α- and β-D-glucosidases, α- and β-D-galactosidases, invertase, β-D-galacturonidase and α-D-mannosidase which hydrolyse simple glycosides and disaccharides. Active polysaccharide-degrading enzymes are limited to α-amylase, laminarinase and 'pectinase'. The extracts may also be a useful source of nigeranase, lichenase, mannodextrinase and polygalacturonase, but not of cellodextrinase and xylanase.

As indicated in Table 1, transglucosylation occurred only with certain β-glucosidases under the conditions used for the survey.

pH-*dependency and relative activities of some seedling carbohydrases.* A freeze-dried seedling extract (A), prepared as described above and containing 52 % of protein, was used for a general survey of the pH-dependency of some of the carbohydrase activities. The digestion conditions are described in the Experimental section. The pH optima, shown in Table 2, were generally about $5{\cdot}0 \pm 0{\cdot}5$.

Table 1. *Qualitative survey of the carbohydrases of alfalfa leaf, stem, seedling and seed*

Oligosaccharide hydrolysis was estimated by visual comparison: 0, no hydrolysis; W, 5–25%; M, 25–60%; S, over 60%. Polysaccharide hydrolysis was estimated by visual comparison of chromatograms of similar quantities of digest: 0, no product visible; W, trace; M, medium; S, intense spot of product.

Substrate	Hydrolysis by extracts: Leaf	Stem	Seedling	Seed
α-Glucosides:				
Methyl α-D-glucoside	0	0	W	W
Nigerose	M	M	M	—
Maltose	M	M	M	W
Isomaltose	W	W	W	W
Turanose	W	0	0	0
β-Glucosides:				
Methyl β-D-glucoside	W	W	W	W
Phenyl β-D-glucoside	W	W	W	W
Laminaribiose	M	S	S	M
Cellobiose	M	M	S*	M*
Gentiobiose	W*	M*	M*	M*
Arbutin	W	W	M	W
Amygdalin	W	W	M	W
Salicin	W	W	M	W
α-Galactosides:				
Melibiose	M	M	S	S
Raffinose	S	M	S	S
Alfalfa-seed galactomannan	W	W	M	W
β-Galactosides:				
Methyl β-D-galactoside	W	M	S	M
Lactose	M	S	S	S
α-Mannosides:				
Methyl α-D-mannoside	W	W	W	W
Sucrose types:				
Sucrose	M	M	S	M
Melezitose	W	W	W	W
Polysaccharide types:				
α-Glucans:				
Starch	S	S	S	S
Nigeran (soluble, acid-degraded)	M	M	W	W
Isolichenin	W	W	W	W
Dextran	0	0	—	—
β-Glucans:				
Laminarin	S	S	S	S
Cellodextrin	W	M	W	W
Lichenin	W	W	S	W
Others:				
Galactan	0	W	W	W
Mannodextrin	W	W	M	W
Inulin	0	W	0	0
Levan	0	W	0	0
Esparto xylan	W	W	W	W
Alfalfa xylan	—	—	W	—
Pectin	S	S	S	S
Alfalfa ammonium pectate	—	—	M	—
Digalacturonic acid	—	S	—	—

* Small quantities of other oligosaccharides were formed showing that some trans-β-glucosylation had taken place.

Table 2. pH *optima and relative activities of seedling carbohydrases*

Seedling preparation contained 52% of protein; the compositions of the digests are described in the text. pH optima values represent the mid-point of peaks covering about ±0·3 pH unit.

Substrate	pH optimum	Hydrolysis (%/hr./mg. of preparation)
α-D-Glucosides:		
Nigerose	4·8	0·37
Maltose	4·8	0·48
Isomaltose	4·8	0·13
Methyl α-D-glucoside	—	< 0·01
Turanose	—	< 0·01
β-D-Glucosides:		
Methyl β-D-glucoside	5·5	0·13
Phenyl β-D-glucoside	4·8	0·63
Laminaribiose	5·1	0·90
Cellobiose	5·3	0·48
Gentiobiose	5·0	0·67
α-D-Galactosides:		
Melibiose	5·0	0·81
Raffinose	5·0	2·40
Galactomannan	4·5	0·36
β-D-Galactosides:		
Methyl β-D-galactoside	4·5	0·75
Lactose	4·8	1·15
Sucrose types:		
Sucrose	6·0	1·25
α-D-Mannosides:		
Methyl α-D-mannoside	5·5	0·12
α-Glucans:		
Starch	5·1	1·47
Nigeran	4·8	0·11
Isolichenin	4·5	0·03
β-Glucans:		
Laminarin	5·5	1·25
Lichenin	5·4	0·35

The activity of the seedling extracts is not due to the presence of contaminating microbial enzymes; for example, the weak activity towards substrates such as methyl α-D-glucoside, methyl α-D-mannoside, melezitose, isolichenin, xylan and galactomannan was the same whether the extracts were prepared from seedlings germinated in the presence or absence of a mixture of antibiotics. Moreover, as shown in Table 3, precautions to prevent microbial growth did not diminish the activity of seedling extracts.

From the pH–activity curves the percentage hydrolysis of a substrate/hr./mg. of added seedling preparation at the optimum pH has been calculated. These values, which represent the relative activities of the enzymes, are also shown in Table 2. However, though the substrate concentrations were the same, the concentration of hydrolysable bond varied.

Enzyme properties and source. A full comparison between the pH–activity curves of seedling and

other enzymes has not been made. However, the pH optima of maltase, cellobiase, lactase and α-amylase have been determined for a leaf extract, and found, in each case, to be identical with those of seedling enzyme.

Action of seedling α-galactosidase action on galactomannan. Hydrolysis of alfalfa-seed galactomannan (containing 46 % of galactose) in a digest containing 8 ml. of galactomannan (5 mg./ml.), 4 ml. of seeding preparation (4 mg./ml.) and 4 ml. of buffer, pH 4·7, was followed by determination of reducing power. The reducing powers, expressed as galactose, at 0, 25, 44 and 68 hr., indicated 2·9, 30·8, 43·7 and 46·8 % hydrolysis of the polysaccharide. The hydrolysis curve tended to be asymptotic to a line at 100 % galactose release, but, in fact, chromatographic examination of the products at 44 and 68 hr. revealed small but increasing quantities of mannose. No mannose was detectable at 21 hr. (26 % hydrolysis).

Table 3. *Comparison of the carbohydrase activities of extracts of seedlings germinated in the presence and absence of antibiotics*

Digests contained substrate (0·25 %), 1 ml. of buffer, pH 5·2, and 1 ml. of enzyme preparation (2 mg./ml.) in a total volume of 4 ml., and were incubated for 22 hr. The extracts were of seedlings germinated in the presence (S) and absence (A) of antibiotics. Activities are expressed relative to that against maltose, which for extracts S and A amounted to 0·70 and 0·63 % hydrolysis/hr./mg. of preparation respectively. Extract S contained 42 % of protein.

Substrate	Activity of extract S	Activity of extract A
Maltose	1·00	1·00
Nigerose	0·82	0·73
Isomaltose	0·27	0·37
Cellobiose	0·89	0·81
Melibiose	2·33	1·43
Sucrose	5·00	1·53
Laminarin	0·99	1·08
Lichenin	0·60	0·73
Lactose	2·10	2·17
Nigeran	0·36	0·36

Table 4. *Effect of germination on seed α-galactosidase*

Protein solutions (1 mg. in 1 ml.) were incubated with substrate (10 mg. in 2 ml.) and buffer, pH 4·5 (1 ml.), for 22 hr.

Germination time (days)	Yield of protein from 25 g. batches of seed (mg.)	Percentage hydrolysis of substrates	
		Melibiose	Galactomannan
0	127	11·4	3·4
2	116	11·2	3·6
3	192	23·7	18·9
4	175	28·7	27·0
5	198	26·6	16·9

Increase with germination. Seeds were extracted by the standard procedure at progressive stages during germination (0, 2, 3, 4 and 5 days). The yield of extractable protein was variable (Table 4) but each solution was adjusted to a concentration of 1 mg./ml. before incubation with substrate. The increasing degree of hydrolysis of both melibiose and galactomannan (Table 4) shows that a true increase of α-galactosidase occurs during germination. The effect was maximal at 4 days.

Action of polysaccharases. Studies of the action of the polysaccharases of alfalfa, except for the amylase, were limited to an investigation of the products of hydrolysis. The amylase was also studied by the decrease in iodine-staining power of soluble starch. Thus the pH–activity curve of the amylase is independent of the presence of α-glucosidase. Chromatographic examination showed that glucose and maltose were the main products of amylase action, but the latter was also attacked by the active α-(1→4)-glucosidase (maltase). During the hydrolysis of polygalacturonic acid and soluble nigeran, only monosaccharide products were obtained at all stages of the hydrolysis. During the hydrolysis of laminarin and xylan, oligosaccharides were detected at intermediate stages of the reaction. It is likely that a mixture of enzymes is operating in these cases. The hydrolysis of mannodextrin and cellodextrin gave mannose and glucose, but no conclusion on polysaccharase action can yet be drawn.

Phosphatases and sugar phosphate isomerases. Glucose 1-phosphate, fructose 6-phosphate and fructose 1,6-diphosphate (each hexose-free) were incubated with extracts under the conditions used for the main chromatographic survey. Glucose 1-phosphate was hydrolysed to glucose, and the fructose phosphates yielded mixtures of glucose and fructose. The presence of both phosphatases and hexose phosphate isomerases is indicated.

DISCUSSION

This survey shows that extracts of alfalfa contain a wide range of carbohydrases, the general complement being similar to that present in ungerminated cereals (Anderson, Cunningham & Manners, 1964) and certain marine algae (Duncan, Manners & Ross, 1956). The range of glycosidases found includes those reported by earlier workers (cited in the introduction), though some activities are weak. For example, the α-D-mannosidase in seed extract was found by Levvy & McAllan (1962) to be very active. However, these workers used *p*-nitrophenyl α-D-mannoside as the substrate; the electronic structure of this compound is such that protonation of the glycosidic oxygen atom, the first stage in acidic hydrolysis (Capon & Overend, 1960) and

probably in enzymic hydrolysis of glycosides, is favoured. The reverse is true for methyl α-D-mannoside.

The pH optima of the seedling carbohydrases are generally about pH 5·0 ± 0·5. Although no comparable survey of any other plant tissue has apparently been reported, the pH optima of many plant carbohydrases (e.g. almond β-glucosidase, malt α-amylase, cereal laminarinase, algal xylanase) are in this range. The control experiments reported on p. 547 and Table 3 show that the activity of the seedling extracts is not due to contamination with carbohydrase-producing microorganisms.

Enzymes acting on α-glucosidic linkages include α-amylase, which occurs widely in terrestrial green plants, and an α-glucosidase that hydrolyses maltose, nigerose and isomaltose at rates in the proportions 1·0:0·77:0·27. The marked activity towards nigerose is of interest since it has not hitherto been reported in the Spermatophyta. Although nigerase activity has been found in animal and fungal enzyme preparations (Larner & Gillespie, 1956; Pazur & Kleppe, 1962), the only report of its activity in higher plants appears to be that of Peat & Rees (1961), who detected nigerase activity in extracts of *Porphyra umbilicalis*. The rarity of such reports is probably due to the general unavailability of substrates containing α-(1→3)-glucosidic linkages rather than to the above being unique sources of α-(1→3)-glucosidase. We have reported (Hutson & Manners, 1963) that nigerase activity generally parallels that of maltase in extracts from 12 common plants. Lukomskaya (1962) has measured maltase, nigerase and isomaltase in rabbit-liver extracts; the observed proportions (1·00:0·75:0·22) are remarkably similar to our values for alfalfa.

The degradation of soluble nigeran, a linear polymer of D-glucose units joined alternately by α-(1→3)- and α-(1→4)-linkages (Barker, Bourne & Stacey, 1953), is unlikely to be due to a single polysaccharase action, but rather to the combined and alternate action of maltase and nigerase. The low activity towards natural undegraded isolichenin (a linear polymer similar to nigeran) confirms this.

The general pattern of enzymic degradation of cellulose and laminarin-type polysaccharides [β-(1→4) and β-(1→3)-linkages respectively] involves the random action of a polysaccharase and a stepwise action of one or more β-glucosidases (see, e.g., Duncan *et al.* 1956; Anderson *et al.* 1964). Alfalfa preparations show strong laminarinase and laminaribiase activities but only limited action on cellodextrin. The lichenase activity is probably due to laminarinase (Perlin & Suzuki, 1962; Cunningham & Manners, 1964). Extracts of several other higher plants show marked β-(1→3)-glucanase activity (Clarke & Stone, 1962). The β-glucosidase component was the only activity that showed transglycosylation properties when incubated with 1 % substrate solution. The transglucosylase action of seedling extract on cellobiose resulted in the synthesis of gentiobiose, laminaribiose and a mixture of trisaccharides, predominantly 6^2-*O*-glucosylcellobiose (Hutson, 1964).

The α-galactosidase is of particular importance because of the presence of galactomannan and the raffinose–stachyose series of oligosaccharides in the seed. There is an increase in α-galactosidase during germination of the seed, and on incubation with galactomannan there is a preferential hydrolysis of the galactose side chains. The weak mannodextrinase activity is probably due to a mannanase that does not begin to liberate mannose from the galactomannan until about half the galactose has been removed. Coffee beans contain enzymes with a similar specificity (Courtois *et al.* 1958).

SUMMARY

1. The carbohydrase activities of extracts of alfalfa leaves, stems, seeds and seedlings have been compared. The extracts show a similar degree and specificity of activity towards various α- and β-glycosides, α- and β-glucans and hemicelluloses.

2. The highest activity is shown towards maltose, laminaribiose, sucrose, raffinose, melibiose, lactose, starch, laminarin and pectin.

3. The effect of pH on the hydrolysis of 20 substrates by the seedling extract was examined. In most instances the pH optimum was about 5.

4. During germination of the seeds there is a marked increase in α-galactosidase activity.

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Variations in Tissue Contents of Coenzyme A Thio Esters and Possible Metabolic Implications

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Coenzyme A is involved in the metabolism of both carbohydrates and fatty acids. It is well established that these two aspects of cellular metabolism are closely interrelated; for example, ready availability of carbohydrate leads to rapid rates of fatty acid synthesis and esterification, whereas conversely high fatty acid concentrations spare the oxidation of glucose and favour gluconeogenesis. Carbohydrate and fatty acid catabolism both yield acetyl-coenzyme A, whereas fatty acids take part in very few biological reactions without prior formation of their coenzyme A thio esters. In view of the central metabolic role of coenzyme A it was thought profitable to investigate the contents of its derivatives *in vivo* in various circumstances, and to consider the metabolic implications of any variations found.

More specifically, interest in two problems led to this work. The first was the profound effect of dietary and other conditions on the rate of fatty acid synthesis in tissues (see Fritz, 1961; Masoro, 1962). For example, Hill, Webster, Linazasoro & Chaikoff (1960) found that oral administration of fat to rats 1–2 hr. before killing greatly impaired fatty acid synthesis in liver slices. The possibility that an intracellular inhibitor of fatty acid synthesis might be found was strengthened by the finding of Korchak & Masoro (1962) that, after starvation, fatty acid synthesis in liver slices was much more reduced than were the activities of the relevant enzymes as measured in dilute cell-free extracts. It was decided to investigate the possibility that the lipogenic inhibitor, perhaps formed by the liver microsomal fraction (Masoro & Porter, 1960), was in fact long-chain acyl-coenzyme A.

The second approach was an investigation of the possibility that the increased rate of fatty acid oxidation in tissues of starved and diabetic animals, usually attributed to an excessive fatty acid supply (see Fritz, 1961), is associated with an elevated intracellular content of long-chain acyl-coenzyme A.

Preliminary reports of parts of this work have been presented elsewhere (Tubbs & Garland, 1963; Garland & Tubbs, 1963; Tubbs, 1963).