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Studies on Dextrans and Dextranases

2. THE ACTION OF MOULD DEXTRANASES ON MODIFIED ISOMALTODEXTRINS AND THE EFFECT OF ANOMALOUS LINKAGES ON DEXTRAN HYDROLYSIS*

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Studies on the degree and types of branching of various dextrans have been carried out by a variety of chemical methods, e.g. methylation, periodate oxidation and cuprammonium complex formation (Neely, 1960). There has been, however, no systematic attempt to study the structures of dextrans by enzymic methods such as those used by Manners (1959) and Bines & Whelan (1960) for glycogens and starches. Dextranases have been obtained from a variety of sources. Exodextranases have been obtained mostly from animal sources, e.g. various types of mammalian tissue, the intestine of *Helix pomatia* and hog intestine. Mould and bacterial sources usually yield endodextranases. The field has been fully reviewed up to 1959 by Fischer & Stein (1960).

Bailey, Hutson & Weigel (1961) reported a detailed study of the action of *Lactobacillus bifidus* dextranase on the branched dextran of *Leuconostoc mesenteroides* (Birmingham strain). We have now examined the mechanism of action of two mould dextranases.

The *Penicillium* dextranases have been chosen for further study because, when the moulds are grown in media containing dextran, culture filtrates containing very high endodextranase activities are readily obtainable. Inducible production of dextranase by certain of these moulds when grown on *L. mesenteroides* (NRRL B-512) dextran was first noted by Tsuchiya, Jeanes, Bricker & Wilham (1952). The resulting extracellular dextranases were shown to cleave dextran randomly to glucose, isomaltose and isomaltotriose. Highly branched dextrans were not readily hydrolysed by these preparations, but no detailed studies were reported.

The availability of essentially unbranched dextran from *Streptococcus bovis* (Bailey, 1959) presented the possibility of inducing the production of a mould dextranase virtually uncontaminated by other carbohydrases, as the substrate contained only α-1→6-linkages. This has in fact been realized and the production, properties and action of two mould dextranases on oligosaccharides and branched dextrans are now reported.

* Part 1: Bailey, Hutson & Weigel (1961).

EXPERIMENTAL

Organisms. Streptococcus bovis (strain I), used for the production of virtually unbranched dextran, was obtained from the National Institute for Research in Dairying, Shinfield, Reading. *Penicillium lilacinum* [strains I.M.I. 27830 (NRRL 895) and I.M.I. 79197 (NRRL 896)] and *Penicillium funiculosum* [strains I.M.I. 79195 (NRRL 1132) and I.M.I. 40235 (NRRL 1768)] were obtained from the Commonwealth Mycological Institute, Ferry Lane, Kew, Surrey.

Dextrans. Most dextrans were from our Departmental Collection. Others were isolated from cultures on sucrose of *Leuconostoc mesenteroides*, the particular strains of which were obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen. *S. bovis* dextran, used as substrate for the moulds, was isolated from a 16 l. culture as described by Bailey & Oxford (1958).

Dextranase production. The moulds were inoculated into a

sterile medium containing *S. bovis* dextran (1%, w/v) and Marmite Co. yeast extract (1%, w/v). The cultures were shaken at 28° for 4 days. Each mould was taken through five subcultures of the same composition. The washed mycelia were then introduced into six 200 ml. batches of media in Erlenmeyer flasks and shaken at 28° for 6 days. Solid material was removed by centrifuging at 3000*g*. The culture fluids were then made 5 mM with respect to sodium citrate, pH 6·0, and dialysed against four changes of 5 mM-sodium citrate, pH 6·0, at 0°. The fluids were then centrifuged at 6000*g* and freeze-dried to brown powders. Yields were about 5 g./1·2 l. of culture fluid.

Dextranase activities. The method used was similar to that of Tsuchiya *et al.* (1952) except that digests were carried out in 0·1 M-sodium citrate buffer and 1 mg. of enzyme preparation was used instead of 1 ml. of culture filtrate. Reducing powers were determined by the method of Shaffer & Hartmann (1921), with isomaltose as a standard. One unit of enzyme is defined as the amount that will produce 1 m-mole of isomaltose monohydrate from *S. bovis* dextran in 1 hr. at 40° under the conditions described above.

Dextran–dextranase digests. Digests were prepared from dextran solutions (100 mg. in 10 ml. of water), 0·2 M-sodium citrate buffer, pH 5·0 (20 ml.), and dextranase (various amounts in 10 ml. of water), and incubated at 37° under toluene.

Variations of dextranase activities with digest conditions were measured at the above concentrations, varying the pH of the buffer and the incubation temperature. Liberation of reducing sugar was measured by the method of Shaffer & Hartmann (1921).

Oligosaccharides. Isomaltodextrins, i.e. oligosaccharides of the isomaltose series, were isolated from a partial hydrolysate of *S. bovis* dextran according to the method of Turvey & Whelan (1957). Isomaltodextrinols, i.e. oligosaccharides of the isomaltose series in which the reducing glucose unit is converted into sorbitol, were made by reduction of the corresponding isomaltodextrins with potassium borohydride (potassium tetrahydroborate) (Bragg & Hough, 1957). 6-*O*-α-Isomaltotriosylfructose (isomaltotetraulose) was made by the epimerization of isomaltotetraose according to the method of Avigad (1959). Other oligosaccharides were from our Departmental Collection, except methyl α-isomaltotrioside for which we are indebted to Dr A. Jeanes. The purity of the carbohydrates was checked by paper chromatography.

Oligosaccharide–dextranase digests. Oligosaccharide (about 10 mg.) in 0·2 M-citrate buffer, pH 5·0 (1 ml.), was incubated with dextranase (2 mg. in 1 ml. of water) at 37° for 16 hr. Digests were de-ionized with Amberlite resin IR-120 (H^+ form) followed by Amberlite resin IR-45 (OH^- form), concentrated in a vacuum desiccator and analysed by paper chromatography and ionophoresis in molybdate solution.

Paper chromatography. The upper layer of an ethyl acetate–pyridine–water mixture (2:1:2, by vol.) was used as a solvent. Compounds were detected with acetone–silver nitrate–ethanolic sodium hydroxide (Trevelyan, Procter & Harrison, 1950). Urea–phosphoric acid (Wise, Dimler, Davis & Rist, 1955) was used for the detection of ketoses. Aniline–diphenylamine–phosphoric acid (Schwimmer & Bevenue, 1956) was used for the detection of, and distinction between, oligosaccharides with a maltose- (blue spot) and an isomaltose-type reducing group (green spot).

Paper ionophoresis. Ionophoresis was conducted in 0·1 M-molybdate, pH 5·5 (Bourne, Hutson & Weigel, 1959).

RESULTS

Production of dextranase by strains of Penicillium lilacinum *and* Penicillium funiculosum. Two strains of *P. lilacinum* [I.M.I. 27830 (NRRL 895) and I.M.I. 79197 (NRRL 896)] and of *P. funiculosum* [I.M.I. 79195 (NRRL 1132) and I.M.I. 40235 (NRRL 1768)] grew readily in media containing sucrose, the culture fluid exhibiting a very weak dextranase activity. After subculture into media containing *S. bovis* dextran, in which the moulds also grew readily, high dextranase activity was exhibited by the filtrates of the culture fluids. The activity increased to a maximum after incubation for 5 days and did not change in a further four subcultures. However, it dropped to its original very low level when the moulds were again grown in media containing sucrose.

Four freeze-dried dextranase preparations were obtained and their activities (see the Experimental section) determined. The results are shown in Table 1.

Action of dextranases on Streptococcus bovis *dextran.* Digests were incubated for 20 hr., de-ionized and analysed by paper chromatography. Components with R_G values identical with those of glucose, isomaltose and isomaltotriose only were detected. Jeanes, Wilham, Jones, Tsuchiya & Rist (1953) characterized these compounds and found them to be the main products when *P. funiculosum* (NRRL 1132, i.e. I.M.I. 79195) dextranase hydrolysed *L. mesenteroides* (NRRL B-512) dextran. The liberation of reducing sugar with time in a standard digest was followed and is shown in Fig. 1. No significant increase of reducing sugars was observed after about 3 hr., except with *P. funiculosum* (I.M.I. 40235) dextranase.

Effect of pH *and temperature on dextranase activity.* Standard digests, containing *S. bovis* dextran, were incubated for 0·5 hr., except those of

Table 1. *Activities of dextranase preparations*

Experimental details are given in the text.

Dextranase	Organism	Activity (units/mg.)
A	*P. lilacinum* (I.M.I. 79197; NRRL 896)	2·0*
B	*P. funiculosum* (I.M.I. 79195; NRRL 1132)	1·1
C	*P. lilacinum* (I.M.I. 27830; NRRL 895)	1·8
D	*P. funiculosum* (I.M.I. 40235; NRRL 1768)	0·2

* The activity of the culture fluid from which the dextranase preparation was isolated was at least 8·3 units/ml. (based on the volume and weight-yield). The activity of the culture fluid, when the organism was grown in a medium containing sucrose, was 0·90 unit/ml.

P. funiculosum (I.M.I. 40235), which were incubated for 3 hr., at various temperatures and pH values. The hydrolysis was then stopped by boiling for 1 min. and the reducing powers of the solutions were measured. The *P. lilacinum* dextranases possessed maximum activity at pH 4·5–5·5 and 45–50°, and *P. funiculosum* dextranases at pH 4·3–5·0 and 45–50°. The results are in agreement with those of Tsuchiya *et al.* (1952). The dextranases of the two strains of each mould did not seem to differ appreciably from each other, and therefore the dextranase of one strain of each mould was selected for further study. These were dextranase A of *P. lilacinum* (I.M.I. 70197) and dextranase B of *P. funiculosum* (I.M.I. 79195).

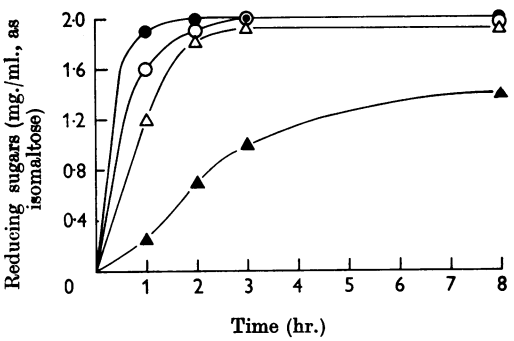

Fig. 1. Liberation of reducing sugars in *S. bovis* dextran–dextranase digests. Identical solutions containing *S. bovis* dextran (about 10 mg./ml.; 10 ml.); 0·2 M-citrate buffer, pH 5·0 (20 ml.), and dextranase (10 mg./ml.; 10 ml.) were incubated at 37°. ○, *P. lilacinum* (I.M.I. 27830) dextranase; ●, *P. lilacinum* (I.M.I. 79197) dextranase; △, *P. funiculosum* (I.M.I. 79195) dextranase; ▲, *P. funiculosum* (I.M.I. 40235) dextranase.

Table 2. *Relative molar yields of glucose, isomaltose and isomaltotriose from isomaltodextrins and dextran on hydrolysis with dextranases A and B*

Substrates were digested with enzymes under standard conditions, and the products separated by paper chromatography and determined with anthrone. Experimental details are given in the text.

Substrate	Products		
	Glucose	Isomaltose	Isomaltotriose
Dextranase A on:			
Isomaltotetraose	16	100	18
Isomaltopentaose	27	100	79
Isomaltohexaose	23	100	47
S. bovis dextran	20	100	59
Dextranase B on:			
Isomaltotetraose	18	100	10
Isomaltopentaose	23	100	41
Isomaltohexaose	33	100	46
S. bovis dextran	38	100	46

Action of the dextranases on isomaltodextrins. The pattern of hydrolysis of isomaltodextrins was studied by incubation with the dextranases under standard conditions. The products (glucose, isomaltose and isomaltotriose) were fractionated by paper chromatography (Lederer & Lederer, 1957) and their relative molar yields determined with anthrone (Yemm & Willis, 1954). The results are shown in Table 2, where they are compared with those obtained from *S. bovis* dextran. The only significant difference between the activities of dextranases A and B seemed to be that the latter produced slightly more glucose and less isomaltotriose from isomaltodextrins than the former. Two digests were prepared containing isomaltotriose (10 mg.) in 0·1 M-citrate buffer, pH 5·0 (2 ml.), and dextranase (10 mg.), and incubated at 37° for 3 days. De-ionization and analysis by chromatography revealed that slow hydrolysis to isomaltose and glucose had occurred. This hydrolysis had proceeded further with dextranase B than with dextranase A. Elution of sugars from chromatograms and determination with anthrone revealed that, under those conditions, dextranase A hydrolysed about 15% of the isomaltotriose, and dextranase B about 30%.

Action of the dextranases on isomaltodextrinols. Isomaltodextrinols were digested under standard conditions; the products were fractionated by paper chromatography and subjected to ionophoresis in molybdate solution, when reducing and non-reducing sugars were quickly separated (Bourne, Hutson & Weigel, 1961). The results are shown in Table 3. Visual estimation of the chromatograms and ionophoretograms showed that approximately equal quantities of the alcohols and reducing sugars of a particular degree of polymerization were formed. Dextranases A and B appeared to hydrolyse the substrates in the same manner.

Table 3. *Products of the action of dextranases A and B on isomaltodextrinols*

Incubations were carried out under standard conditions for 16 hr., and the products separated by chromatography and ionophoresis in molybdate solution. Experimental details are given in the text.

Isomaltodextrinol	Products
Isomaltitol	Unchanged isomaltitol
Isomaltotri-itol	Unchanged isomaltotri-itol and traces of isomaltitol, isomaltose, glucose and sorbitol
Isomaltotetraitol	Isomaltose, isomaltitol and traces of glucose and sorbitol
Isomaltopentaitol	Isomaltotriose, isomaltotri-itol, isomaltose, isomaltitol and traces of glucose and sorbitol
Isomaltohexaitol	Isomaltotriose, isomaltotri-itol, isomaltose, isomaltitol and traces of glucose and sorbitol

Action of the dextranases on other modified isomaltodextrins. Four oligosaccharides were incubated with dextranases under standard conditions. The products were identified by chromatography with the silver nitrate and aniline–diphenylamine reagents. Ionophoresis in molybdate solution, a specific test for 6-*O*-substituted fructoses (Bourne, Hutson & Weigel, 1960), and the urea–phosphoric acid spray were used to detect isomaltulose in the hydrolysate of isomaltotetraulose. It was not evident, from chromatographic analyses, that the pentasaccharide was cleaved preferentially to a particular mixture of di- and tri-saccharide. The results are shown in Table 4.

Action of the dextranases on branched dextrans. Dextranases A and B were separately incubated under standard conditions with a number of dextrans of various types and degrees of branching. All the dextrans (except *S. bovis* dextran as control) were from various strains of *L. mesenteroides*. The reducing sugar liberated was measured as isomaltose at intervals over 10 hr. No significant increase of reducing sugar was observed after incubation for about 3 hr. The average values obtained after incubation for a further 7 hr. are shown in Table 5.

Table 4. *Products of the action of dextranases A and B on isomaltodextrins containing anomalous linkages and moieties*

Experimental details are given in the text.

Modified 'isomaltodextrin'	Products of dextranase action*
6-*O*-α-Isomaltotriosyl-fructose	Isomaltose, isomaltulose
4-*O*-α-Isomaltotriosylglucose	Isomaltose, maltose
4-*O*-α-Isomaltotetraosyl-glucose	Isomaltotriose, panose, isomaltose, maltose
Methyl α-isomaltotrioside	Isomaltose, methyl α-glucoside†

* Traces of glucose were present in the products; however, only the main hydrolysis products, as revealed by the aniline–diphenylamine spray, are shown above.

† Slow reaction with silver nitrate reagent.

DISCUSSION

Production of extracellular dextranases by strains of *P. lilacinum* and *P. funiculosum* is induced when the moulds are grown in media containing the virtually unbranched *S. bovis* dextran. Their general properties do not seem to differ from the preparations elaborated in media containing the branched dextran of *L. mesenteroides* (NRRL B-512) (Tsuchiya *et al.* 1952).

The products of the action of the dextranases on *S. bovis* dextran are glucose, isomaltose and isomaltotriose, isomaltose being the main product. This suggests that isomaltotetraose is the smallest readily hydrolysed isomaltodextrin. This has been shown to be the case, the molecule being hydrolysed principally at the central glycosidic linkage to give isomaltose.

The composition of the hydrolysate of isomaltopentaitol shows that the two non-terminal glycosidic linkages are hydrolysed at similar rates and much faster than the terminal linkages. Likewise the non-terminal glycosidic linkages of the isomaltohexaitol are readily hydrolysed. It can be assumed that the same is true for isomaltopentaose and isomaltohexaose.

When the reducing glucose unit of isomaltotetraose was replaced by sorbitol (6-*O*-α-isomaltotriosylsorbitol), fructose (6-*O*-α-isomaltotriosylfructose), an α-1→4-linked glucose unit (4-*O*-α-isomaltotriosylglucose) or a methyl group (methyl α-isomaltotrioside), hydrolysis still occurred at approximately the same rate and there was no change in the pattern of the hydrolysis. This shows that the dextranases have no preference for a

Table 5. *Liberation of reducing sugars from various dextrans by dextranases A and B*

Incubations were carried out under standard conditions for 10 hr. Experimental details are given in the text.

Origin of dextran	Percentage of linkage present				Percentage of reducing sugar liberated by dextranases (as isomaltose)	
	1→6	1→4	1→3	1→2	Dextranase A	Dextranase B
*S. bovis**	100	—	—	—	96·6	96·6
L. mesenteroides (Birmingham; NRRL B-1375) modified†	98	—	2	—	86·4	—
L. mesenteroides (NRRL B-512)‡	95	—	5	—	87·9	87·5
L. mesenteroides (NRRL B-1383)‡	84	16	—	—	46·3	—
L. mesenteroides (Birmingham; NRRL B-1375)‡	81	6	13	—	44·4	49·2
L. mesenteroides (NCIB 2706)‡	79	3	18	—	22·7	22·7
L. mesenteroides (NRRL B-742)‡	67	21	12	—	12·5	17·4
L. mesenteroides (NRRL B-1399)‡§	65	6	—	29	36·6	—

* Bailey (1959).

† Barker, Bourne, James, Neely & Stacey (1955).

‡ Jeanes *et al.* (1954).

§ Scott, Hellman & Senti (1957).

particular type of chain-end, a result which is in accordance with the properties of endopolysaccharases. It thus seems that the essential requirements for ready hydrolysis to occur are realized in methyl α-isomaltotrioside, where the terminal glucosidic linkage at the 'reducing end' is merely an α-glucosidic linkage. It is therefore probable that the dextranases hydrolyse readily virtually unbranched dextran, isomaltodextrins or modified isomaltodextrins containing not less than three glucosidic linkages, the hydrolysis being principally at glucosidic linkages other than the terminal ones, as shown in Fig. 2.

Small amounts of glucose were found in all cases when dextran or isomaltodextrins were acted on by the dextranases. Similarly, traces of glucose and sorbitol were found when isomaltodextrinols were hydrolysed. As expected from these results, isomaltotriose was hydrolysed only very slowly. Whereas Walker & Whelan (1960) found that starch and maltotetraose represent the two extremes of molecular size on which salivary α-amylase acts rapidly, the extreme molecular sizes for the dextranases are represented by dextran and methyl α-isomaltotrioside. The marked difference in rate of hydrolysis between methyl α-isomaltotrioside and isomaltotriose might be due to such properties as mutarotation of reducing sugars, the electronic structures of the substituents in the terminal glucosidic oxygen, or both.

Anomalous linkages in natural dextrans usually occur as branch linkages and it is likely that they will affect the pattern of enzymic hydrolysis. Table 5 shows that the final amount of reducing sugar liberated is markedly lowered with increasing degree of branching. This indicates that the products from a branched dextran are of greater average molecular size than those from an unbranched dextran. It is likely that these limit dextrins contain the anomalous linkages and will be formed for two reasons: (i) the dextranases are incapable of hydrolysing the anomalous linkages and (ii) the anomalous linkage renders a neighbouring region in the dextran molecule incapable of being hydrolysed by the dextranases. Analysis of the limit dextrins containing the anomalous linkages would aid both structural studies of dextrans and investigations of the mechanism of dextranase action.

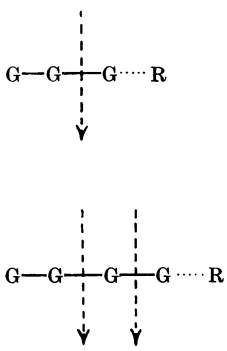

Fig. 2. Action of dextranases on dextran, isomaltodextrins and modified isomaltodextrins. ——, α-1→6-link; · · ·, α-link; G, glucosyl unit; R, glucose, fructose, sorbitol or methyl group; - - - →, principal points of hydrolysis.

SUMMARY

1. Extracellular dextranases are adaptively produced by strains of *Penicillium lilacinum* and *P. funiculosum* grown in media containing the virtually unbranched *Streptococcus bovis* dextran.

2. The products of the action of the dextranases on *S. bovis* dextran are glucose, isomaltose and isomaltotriose, isomaltose being the main product.

3. The dextranases readily hydrolyse virtually unbranched dextran, isomaltodextrins or modified isomaltodextrins containing not less than three glucosidic linkages, the hydrolysis being principally at glucosidic linkages other than the terminal ones.

4. The number of reducing groups liberated from various dextrans by the dextranases is markedly lowered with increasing degree of branching of the dextran molecule.

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The Metabolism of Glyoxylate by Human- and Rat-Liver Mitochondria

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The place of glyoxylate as an intermediate in glycine metabolism has been reviewed by Weinhouse (1955), although its importance in the intact animal is still under discussion (Neuberger, 1961; Liang, 1962*a*, *b*). Nakada & Sund (1958), who studied the decarboxylation of glyoxylate by washed liver homogenates and prepared a partially purified glyoxylate decarboxylase from liver mitochondria, suggested that glyoxylate and L-glutamate condense to form *N*-glyoxyl-L-glutamic acid (*N*-carboxymethylene-L-glutamic acid), which is either hydrolysed to glycine and 2-oxoglutarate or decarboxylated by glyoxylate decarboxylase to *N*-formyl-L-glutamic acid. According to this scheme formic acid and L-glutamic acid are produced by the subsequent hydrolysis of *N*-formyl-L-glutamic acid. Glyoxylate can also be oxidized enzymically to oxalate *in vitro* (Ratner, Nocoto & Green, 1944; Nakada & Weinhouse, 1953*a*; Richardson & Tolbert, 1961; Quayle & Taylor, 1961) and *in vivo* (Weinhouse & Friedmann, 1951).

The present work was designed to investigate the mechanism of glyoxylate metabolism in intact mammalian liver mitochondria. We also attempted to determine whether *N*-formyl-L-glutamic acid was a metabolic intermediate in this system as well as in Nakada & Sund's (1958) system. The cytochrome-oxidase activity of the mitochondria was used as an independent criterion of their biological activity.

Some of the results presented here have been the subject of preliminary communications (Crawhall & Watts, 1960, 1961).

MATERIALS AND METHODS

Melting points are uncorrected and microanalyses (C, H, N) were by Weiler and Strauss, Oxford, and by A. Bernhardt, Mülheim.

All chemicals were of A.R. grade, and water that had been deionized by passage through a mixed-bed ion-exchange column was used. ATP, ADP and cytochrome *c* were obtained from the Sigma Chemical Co., glyoxylic acid from L. Light and Co. Ltd., oxaloacetic acid and 2-oxoglutaric acid from C. F. Boehringer und Soehne (through Courtin and Warner Ltd.). [1-^{14}C]Glyoxylic acid, sodium [1-^{14}C]glyoxylate, 2-[5-^{14}C]oxoglutaric acid, DL-[1-^{14}C]-glutamic acid and [2,3-$^{14}C_2$]fumaric acid were obtained from The Radiochemical Centre, Amersham, Bucks.

Synthesis of [2-^{14}C]*sodium glyoxylate.* [2,3-$^{14}C_2$]Fumaric acid (1 m-mole) was oxidized with OsO_4 and $KClO_3$, and potassium hydrogen [2,3-$^{14}C_2$]tartrate isolated (Weissbach & Sprinson, 1953). An aqueous solution of potassium hydrogen [2,3-$^{14}C_2$]tartrate was passed through a column (5 cm. × 1 cm.) of Dowex 50 (H^+ form) and the [2,3-$^{14}C_2$]tartaric acid in the eluate converted into sodium [2-^{14}C]glyoxylate as described by Radin & Metzler (1955). Yield, based on [2,3-$^{14}C_2$]fumaric acid, was 30%. The product could be converted into the 2,4-dinitrophenylhydrazone in 95% yield, which agrees with the experience of Wild (1953) and Radin & Metzler (1955). Two batches of unlabelled sodium glyoxylate synthesized in this way gave Na 20·2 and 19·9% respectively (weighed as Na_2SO_4) ($C_2H_3O_4Na$ requires Na, 20·2%) and acid $KMnO_4$ titrations that corresponded to 102 and 101% of the theoretical value for $(OHC{\cdot}CO_2{\cdot}Na),H_2O$.

Synthesis of N-*formyl*-L(−)-*glutamic acid.* Two batches of this material were prepared. On the first occasion we followed the procedure of Tabor & Mehler (1955), on a 45 m-moles scale, and obtained a yield of only 7%. The product melted at 108–109°. (Found: C, 41·4; H, 5·1; N, 8·0; $C_6H_9O_5N$ requires C, 41·4; H, 4·6; N, 8·0%). On the second occasion freshly redistilled acetic anhydride (30 ml.) was added dropwise over the course of about 0·5 hr. to formic acid (90 ml., 98/100%) in a three-necked flask fitted with a spiral condenser, a Hg-sealed stirrer and a dropping funnel. The condenser and dropping funnel were fitted with guard tubes containing Anhydrone. Stirring was continued until the temperature of the reaction mixture, which rose initially, had returned to room temperature. The flask was then cooled in ice–NaCl freezing mixture, L(−)-glutamic acid (45 m-moles) was added and stirring continued for 1 hr. The remaining acetic anhydride and