RESULTS

The silage specimens on which data are reported were from grass and grass-clover mixtures ensiled during the summer of 1950 on various farms in the north-east of Scotland, and were taken at random from specimens being analysed here.

It is seen from Fig. 2 that the lactic acid content of silage falls rapidly with rise of pH. This is in accordance with the generally held view that in silage of pH greater than 4·2 the amounts of lactic acid are small. It has not been definitely shown, however, whether or not lactic acid is formed in all silage but is subsequently destroyed in silage of high pH. An investigation into this problem is the subject of further work.

SUMMARY

The use of the procedure of Barker & Summerson (1941) for determining lactic acid is described in its relation to the estimation of this acid in silage.

It is desired to thank Mr John Baxter for his assistance in the technical side of the present work.

REFERENCES

Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
Davidson, J. (1949). *J. Dairy Res.* **16**, 209.
Foreman, F. W. (1920). *Biochem. J.* **14**, 451.
Hirsch-Kauffmann, H. (1924). *Hoppe-Seyl. Z.* **140**, 25.
Karström, K. & Virtanen, A. I. (1937). *Ann. Acad. Sci. Fenn.* **48**, no. 14.
Lieb, H. & Zacherl, M. (1932). *Hoppe-Seyl. Z.* **211**, 211.
Mendel, B. & Goldscheider, I. (1925). *Biochem. Z.* **164**, 163.
Miller, B. F. & Muntz, J. A. (1938). *J. biol. Chem.* **126**, 413.
Russell, J. A. (1944). *J. biol. Chem.* **156**, 463.
Watson, S. J. & Ferguson, W. S. (1937). *J. agric. Sci.* **27**, 1.

Transfructosidation in Extracts of the Tubers of *Helianthus tuberosus* L.

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In the artichoke tuber there is present a mixture of carbohydrates, of which sucrose is the component with smallest molecular weight, and inulin probably the largest (Bacon & Edelman, 1951). Since the only enzyme system which is known to produce a fructose polysaccharide *in vitro* (levansucrase; Hestrin, Avineri-Shapiro & Aschner, 1943) does so from sucrose, it seemed worth while to investigate the possibility that inulin was formed from sucrose, and with this aim in view the action of tuber extracts on sucrose was studied.

The results presented in this paper show that a reaction takes place in tuber extracts by which oligosaccharides are synthesized from sucrose at the expense of fructose residues from inulin and related polysaccharides. The name 'transfructosidation' is proposed for this type of reaction. A preliminary account was given to the Biochemical Society at Edinburgh on 21 July 1950 (Edelman & Bacon, 1950).

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METHODS

The methods were those described by Bacon & Edelman (1951) and Edelman & Bacon (1951).

Dandelion inulin (Thomas Kerfoot and Co. Ltd., Vale of Bardsley, Ashton-under-Lyne) was purified as described by Edelman & Bacon (1951). Sucrose was recrystallized one or more times from 95% aqueous ethanol. Both carbohydrates were shown by paper partition chromatography to be free from other oligosaccharides and free hexoses.

All incubations were carried out in the presence of $CHCl_3$ unless otherwise stated.

RESULTS

Initial investigation

Qualitative experiments. It was assumed that any synthetic activity in the presence of sucrose would lead first to the production of oligosaccharides behaving, on the paper partition chromatogram, like spots 2, 3 and so on (Bacon & Edelman, 1951). Since these substances were already present in the tubers, dialysis was carried out to remove them.

Mash, prepared in November 1949, was dialysed against running cold water for 50 hr., the dialysis sac being periodically agitated by a rocking movement. Two 25 ml. samples of the dialysed material were adjusted to pH values of 5·0 and about 6·5 with 1 ml. M-sodium acetate buffer and drops of 0·2N-sodium hydroxide respectively. Samples (10 ml.) of these preparations were each incubated at 28° with 1 ml. 50% sucrose. Other samples were incubated without added sucrose. Chromatograms of 20 and 45 hr. incubation samples containing added sucrose showed, at both pH values, a spot in position 2 of the artichoke carbohydrate series. The higher pH value gave a rather more pronounced effect as judged qualitatively. Spot 2 did not appear in the controls. Although free glucose and fructose appeared in rather greater amount in the samples incubated with sucrose than in the controls, only small amounts of these sugars were formed. Further experiments, similar to the one described, confirmed that spot 2 appeared in the presence of sucrose, but not in its absence, and showed that it was formed in greater amount at pH values 5·8 and 6·4 than at 4·2 and 7·2. Boiled extract in the presence of sucrose did not give the reaction. Some of the incubated samples in these experiments contained only traces of monosaccharide, although considerable amounts of spot 2 had been formed (see Fig. 1).

Quantitative experiments. The effect was studied in undialysed mash by developing the use of quantitative paper partition chromatography to follow changes in the amounts of the oligosaccharide components. Two samples of 25 g. mash were incubated at 28° with 2·5 ml. 33% sucrose and 2·5 ml. water respectively. No pH adjustment was made. Samples of the liquid strained from the incubated mash through gauze were chromatographed in butanol-acetic acid and the ketose content of the spots estimated. Results are given in Table 1.

A micrometer syringe was not used in applying the 5 μl. samples of liquid to the filter paper in this experiment; a 0·1 ml. graduated pipette was used instead, with consequent loss in accuracy. In spite of this, however, the table shows that there were no major changes in any of the control incubations except in spot *F* (free fructose) and spots 5 to *n*. Spots 2 and 3 in the sample incubated with sucrose, however, showed a very marked increase, and there was a smaller but probably significant increase in spot 4. Spot 1 (sucrose) decreased. Fructose was liberated in similar amounts in both samples. A discrepancy was noticed, namely that whereas the overall increase in spots 2–4 was 261 μg. fructose/20 μl., the disappearance of fructose from spot 1 (sucrose) was only 144 μg./20 μl. It did not seem possible that such a large difference could be caused by experimental error.

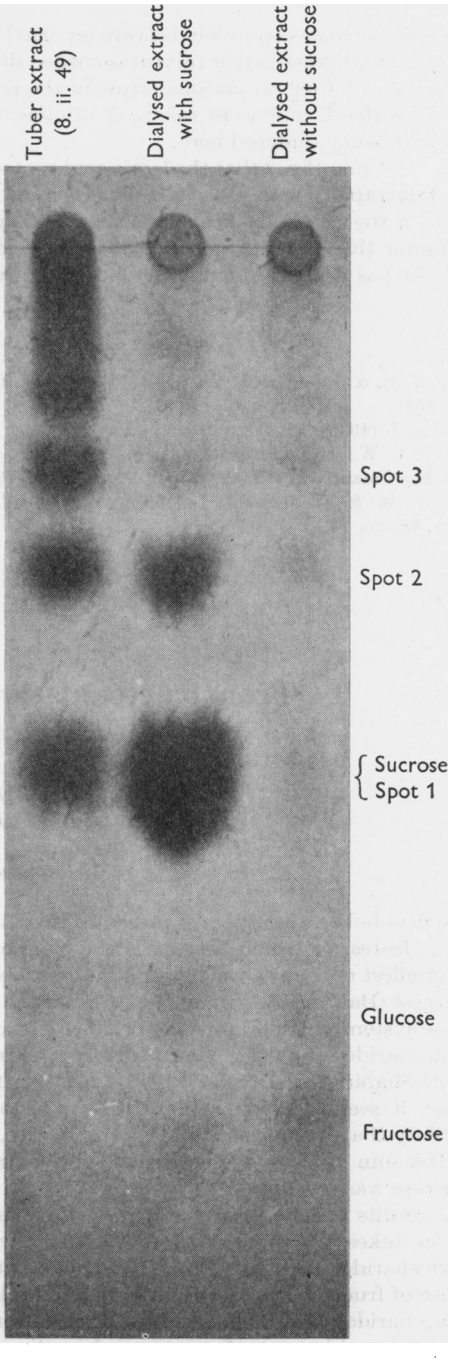

Fig. 1. Extract, prepared December 1949, dialysed 28 hr. against running tap water at 9°. Incubated 21 hr. at 26° with and without 10% added sucrose. Chromatogram run in butanol-acetic for 2 days; sprayed with benzidine-trichloroacetic.

No glucose could be detected qualitatively in this experiment in spite of the sensitivity of the spraying reagent used (benzidine-trichloroacetic) which would have detected as little as 5 μg. glucose/20 μl. under the conditions used. A similar experiment to the one described, in which the presence of free glucose was investigated qualitatively and quantitatively, confirmed this result. Although the estimations of glucose were not very satisfactory (cf. Bacon & Edelman, 1951), it appeared from these and from the qualitative results that dismutation between sucrose molecules according to the equation

$$2\,(\text{glucose-fructose}) \rightarrow \text{glucose-(fructose)}_2 + \text{glucose}$$

could not account for the observations.

Table 1. *Incubation of tuber whole mash with added sucrose*

(Mash, prepared November 1949, incubated at 28° with 0·1 vol. 33% sucrose (w/v). No buffer added, pH about 6·5. Results of quantitative chromatographic analysis expressed as μg. ketose/20 μl. incubate, strained through gauze.)

	Time of incubation			
	0 hr.		27 hr.	
Spot	Sucrose added	Control	Sucrose added	Control
F	—	—	154	176
1	450	110	306	92
2	132	110	288	110
3	135	128	214	135
4	132	121	158	119
5 to *n*	1110	1050	800	920
Total	1959	1519	1920	1552

Elimination of extraneous causes. At this stage it was thought advisable to eliminate the possibility that the effects observed were due to contaminating bacteria, or to purely physical effects on the paper chromatogram.

(*a*) *Bacterial contamination.* In order to eliminate a heavy initial inoculum of micro-organisms tubers were scrubbed thoroughly with water and then with soap and water. They were peeled, washed with water, then with 70% ethanol, and the surface layers removed again. The remaining material was extracted in the usual way, chloroform being added at the start of disintegration in the Waring blender. Sucrose solutions saturated with chloroform were made up immediately before the tubers were extracted. Incubation was started a few minutes after the preparation of the extracts and stopped 2·5 or 5 hr. (Table 2) later. Chloroform was present throughout the incubations. A significant increase in spot 2 occurred in both cases. It is unlikely that bacteria caused the effect under these conditions during so short a period of incubation.

Earth chipped from the same batch of tubers was incubated at 37° with 7% sucrose in 0·004M-ammonium chloride. No material with R_F lower than sucrose was detected even after incubation for 52 hr. in the absence of chloroform. Traces of free glucose and fructose appeared under these conditions, but were not formed in the presence of chloroform.

Table 2. *Activity in absence of significant bacterial inoculum*

(See text for details. Extract, prepared January 1950, incubated at 30° with 0·125 vol. 40% sucrose (w/v) or water. No buffer added; pH about 6·5. Values expressed as μg. ketose/20 μl. incubate.)

	Time of incubation			
	0 hr.		5 hr.	
Spot	Sucrose added	Control	Sucrose added	Control
1	550	121	540	132
2	121	108	216	126
3	139	139	142	140
4 to *n*	1160	1160	1180	1130

All extracts used subsequently were made from peeled tubers washed thoroughly in water.

(*b*) *Physical interference on chromatograms.* The increase in spots 2–4 was not due to a physical effect of large amounts of sucrose on the chromatographic separation. Zero hour samples showed no significant differences from the controls (see Tables 2, 4, 8 and 9), boiled extract showed no increase of spot 2 in the presence of added sucrose (Table 3), and during the reaction there was a progressive increase of spot 2 with time of incubation (see below).

Table 3. *Effect of added sucrose on the chromatographic analysis of boiled extract*

(Sucrose added to 'standard substrate' (Edelman & Bacon, 1951).)

	μg. ketose/20 μl. solution	
Spot	Standard substrate with added sucrose	Standard substrate alone
1	485	150
2	150	146
3	160	160
4 to *n*	960	960

Effect of dialysis. Dialysis reduced the activity considerably, but did not destroy it entirely. This effect, first noticed qualitatively, is shown in Table 4, which shows also that dialysis for 53 hr. did not eliminate all the polysaccharide in the extract, the lower spots in the case of the dialysed extract incubated without sucrose probably being formed by the action of the hydrolytic system on the residual polysaccharide.

As compared with spot 2 glucose was formed in significant amounts in the presence of sucrose. However, not all dialysed extracts produced free glucose under these conditions.

Table 4. *Effect of dialysis on the reaction*

(Extract, prepared December 1949, dialysed 53 hr. at 9°. 1 vol. 50% sucrose incubated at 30° with 9 vol. of the dialysed liquid. No buffer added; pH about 6·7. Values expressed as μg. ketose/20 μl. incubate, except for 'spot *G*' which is expressed as μg. reducing substances (RS; calculated as hexose)/20 μl.)

	Time of incubation (hr.)											
	0		4		10		23		50		78	
Spot	Sucrose added	Control	Sucrose added	Control	Sucrose added	Control	Sucrose added	Control	Sucrose added	Control	Sucrose added	Control
F	46	56	57	62	77	79	91	112	139	130	176	156
G	0	0	0	0	0	0	35	0	44	0	58	0
1	500	11	505	11	525	20	432	35	424	23	490	26
2	225	245	28	9	49	12	66	14	57	23	44	13
3 to *n*			218	218	208	203	175	183	139	144	122	122
Total ketose	771	312	808	300	859	314	764	344	759	320	832	317

Table 5. *Restoration of activity to dialysed extract*

(See text for details. The pH of all incubations lay between 6·44 and 6·52. Values expressed as μg. ketose/20 μl. incubate.)

	Spot 2			Spot 3			
Incubation	Before	After	Increase	Before	After	Increase	Total Increase
1	73	146	73	75	81	6	79
2	74	145	71	75	79	4	75
3	64	129	65	62	68	6	71
4	66	126	60	62	70	8	68
5	123	203	80	110	132	22	102

Addition of boiled undialysed extract completely restored the activity. Two separate dialyses of the same extract were carried out for 9 and 22 hr. respectively against running water at 9°. Glass tubes containing undialysed extract were suspended in the running water for these periods. The dialysed extract increased in volume from 40 to 46 ml. (9 hr.) and from 40 to 49 ml. (22 hr.). The undialysed samples were diluted similarly. A sample (10 ml.) of each of the four extracts was placed in a boiling-water bath for 5 min. and then cooled. Five incubates were set up: (1) undialysed extract (2·5 ml.) + boiled dialysed extract (2·5 ml.) (9 hr. dialysis); (2) boiled undialysed extract (2·5 ml.) + dialysed extract (2·5 ml.) (9 hr. dialysis). Incubates 3 and 4 were set up, similarly to 1 and 2 but using the 22 hr. dialysed samples. Incubate 5 contained 2·5 ml. undialysed extract + 2·5 ml. boiled undialysed extract. To each incubate was added 1·0 ml. 30% sucrose solution to give an overall concentration of added sucrose of 5%. The results of analysis of the various components after 22 hr. incubation at 30°, given in Table 5, show that dialysed extract in the presence of boiled extract had the same activity as undialysed extract.

Attempts to concentrate the enzymic activity. An extract, prepared January 1950 (pH adjusted to 7 with N-sodium hydroxide), was fractionated between zero and 0·25, and 0·25 and 0·50 ammonium sulphate saturation by adding the solid (A.R.). The precipitates which formed were separated by centrifugation. Aqueous suspensions of these precipitates were dialysed overnight against running water and incubated at 28° with an overall concentration of 25% added sucrose at pH values of 6·8 (in 0·1M-phosphate buffer) and 5·0 (in 0·1M-acetate buffer). Samples taken at zero time showed no carbohydrate material on the chromatogram other than sucrose. In the case of the 0·25–0·50 saturation precipitate, later samples (5, 10, 28 and 53 hr.) showed traces of spot 2, glucose, and fructose, all becoming progressively more distinct in the course of time. These spots were weaker in the pH 5·0 incubate. The incubate containing the zero to 0·25 saturation precipitate showed only the slightest traces of these additional spots, and their appearance was delayed as compared with the 0·25–0·50 saturation precipitate. Even the greatest activity shown in this experiment, however, was negligibly small when compared with that of fresh tuber extracts. Further ammonium sulphate precipitates obtained between 0 and 0·60 saturation showed no detectable formation of spot 2 when incubated with sucrose, but their activity could be restored by adding boiled fresh extract. Such a precipitate, obtained from 300 ml. fresh extract, was taken up in water to give 15 ml., and this solution dialysed for 20 hr. against running cold water. To 4·0 ml. boiled fresh extract were added 0·8 ml. 30% sucrose solution and 1·0 ml. of this dialysed preparation, and to 4·0 ml. unboiled extract were added 0·8 ml. 30% sucrose and 1·0 ml.

Table 6. *Restoration of activity to ammonium sulphate concentrate by addition of boiled extract*

(For details see text. *A*, enzyme concentrate plus boiled extract. *B*, fresh extract. Concentration of added sucrose about 4%. No buffer added; pH about 6·7. Incubated at 30°. Values expressed as μg. ketose/20 μl. incubate.)

	Time of incubation					
	0 hr.		3 hr.		6 hr.	
Spot	*A*	*B*	*A*	*B*	*A*	*B*
F	29	32	47	42	61	47
1	553	553	517	517	480	493
2	88	95	166	142	211	176
3	92	88	103	102	108	109
4 to *n*	805	805	760	746	733	705
Total ketose	1567	1573	1593	1549	1593	1530

water. The mixtures were incubated at 30°. Results of analyses are given in Table 6; they showed that under these conditions the ammonium sulphate precipitate contained considerable activity. These experiments and those described above suggested that a thermostable co-factor was present in active preparations.

As the active system in tuber extracts could not be separated from the carbohydrates by dialysis or ammonium sulphate precipitation, further investigation was, of necessity, carried out on fresh extracts.

Further investigations of fresh extracts

Effect of varying pH *on the reaction.* The optimum lay between pH values 6·0 and 6·5 (see Table 7). As the pH of fresh extracts was in the neighbourhood of 6·5 no buffer was added in subsequent experiments.

Progress of the reaction with time. This is shown in Table 8. The following points were noticed: (1) Fructose is liberated in similar amounts both in the presence and absence of added sucrose. Although no figures were obtained for free glucose, this sugar was present in traces only (less than 5 μg./20 ml. incubate) and did not increase noticeably either in the presence of added sucrose nor during the progress of the incubation. (2) Ketose in spot 1 (sucrose) disappeared more rapidly in the incubate with added sucrose than in the control. This disappearance increased with increasing time of incubation.

Table 7. *Effect on* pH *on the increase in spot* 2

(Extract prepared 28 February 1950. pH adjusted with drops of N-HCl or N-NaOH. 1·0 ml. 30% sucrose was added to 5·0 ml. samples. Incubated at 30° for 4 hr. The amounts of spot 2 are represented by μg. ketose/20 μl.; the initial values at all other pH values are assumed to be equal to that for pH 7·0.)

	pH				
	7·0	6·5	6·0	5·5	5·0
Before	142	—	—	—	—
After	197	241	237	220	192
Increase	55	99	95	78	50

Table 8. *Progress of the reaction with time*

(Extract, prepared March 1950, incubated at 30° with 0·2 vol. 30% sucrose (w/v) or water. No buffer added. pH before incubation: of sucrose incubate 6·82, of control 6·84; after 20 hr. incubation: both 6·75. Values expressed as μg. ketose/20 μl. incubate.)

	Time of incubation (hr.)						Change in 18 hr.
Spot	0	1	2	4	7	18	
			(*a*) With added sucrose				
F	24	29	36	45	53	92	+ 68
1	666	654	642	636	606	570	− 96
2	150	178	201	243	278	357	+207
3	165	176	168	182	176	194	+ 29
4 to *n*	1214	1204	1158	1158	1114	1068	−146
Total ketose	2219	2244	2205	2264	2227	2271	—
			(*b*) Control				
F	23	30	37	46	57	102	+ 79
1	156	153	150	150	148	148	− 8
2	133	144	147	165	156	166	+ 33
3	155	159	155	167	169	184	+ 29
4 to *n*	1220	1204	1220	1200	1296	1120	−100
Total ketose	1687	1690	1769	1728	1826	1720	—

(3) The ketose content of spot 2 increased throughout the time of incubation, very much more in the incubate with added sucrose than in the control. (4) There was no difference between the rates of increase of the ketose contents of spot 3 in the two incubates. (5) There was some indication that the ketose content of spots 4 to *n* was decreasing more rapidly in the presence of added sucrose than in the control. (The fluctuation of the ketose content of this group of spots was much larger in absolute terms than that of the lower spots because much larger total amounts were being estimated; measurement of the decrease, which was of the same order as the change in spot 1, was therefore subject to a much larger experimental error.) (6) The amount of combined fructose which disappeared from spot 1 approximated fairly closely to half the fructose appearing in spots 2 and 3 together.

From (5) and (6) above, the possibility was suggested that the material causing the increase in spots 2 and 3 was derived partly from spot 1 and partly from higher spots.

Effect of varying sucrose concentration. The effect of varying concentrations of sucrose on the reaction was therefore investigated in an attempt to establish a more reliable quantitative relationship between the changes in ketose content of the various components of the system. The results of such an experiment are given in Table 9, which shows good agreement between the total ketose estimated before and after incubation, the greatest discrepancy being about 3% and the average 1%. Fructose was liberated in similar amounts at all levels of added sucrose, there being a slight decrease with increasing concentration of added sucrose at the higher levels. The increase in ketose content of spot 3 was also similar at all levels of added sucrose concentration except the two highest. These variations in spots *F* and 3 are sufficiently small to have little effect on the calculations of the changes in spots 1, 2 and 4 to *n*, in making which it has been assumed that the ketose appearing in spots *F* and 3 was derived ultimately from spots 4 to *n*. In the table, therefore, all material higher than spot 2 has been treated as a single entity, namely spots 3 to *n*.

When the amount of spot *F* is added to the values for spots 3 to *n* there remains still a significant amount of ketose to be accounted for (Table 9*b*, iii), an amount increasing with increasing sucrose concentration, and approximately equal to the corresponding decrease in spot 1 (Table 9*b*, iv, v). It should be noted that small percentage errors in analysis would cause very large errors in the ratios given in (v), particularly at the extremes of sucrose concentration, since with no added sucrose the error in measuring spots 3 to *n* is large in comparison with

Table 9. *Effect of varying sucrose concentration on the reaction*

(Extract prepared March 1950. Incubated at 28° with 0·2 vol. sucrose solution of appropriate concentration. No buffer added; the pH values of all incubates lay between 6·53 and 6·57. All values expressed as μg. ketose/20 μl. incubate.)

		Concentration of added sucrose as percentage (w/v)													
		Zero		0·5		1·0		1·5		3·0		5·0		8·5	
(*a*) Analyses:															
Spot *F*		33*	79†	35	75	35	81	36	79	36	77	35	74	33	69
1		155	144	203	178	248	213	299	252	427	345	595	485	957	910
2		137	163	137	185	141	212	143	236	145	278	152	312	149	355
3		138	155	135	162	140	162	144	171	138	164	146	162	155	160
(4 to *n*)		1095	1007	1095	997	1083	986	1075	957	1099	957	1095	925	1077	947
Total		1558	1548	1605	1597	1647	1654	1696	1695	1845	1821	2023	1958	2371	2441
(*b*) Calculations:															
Increase in spot *F*	(i)	46		40		46		44		41		39		36	
Decrease in spots (3 to *n*)	(ii)	70		71		75		91		116		154		125	
Decrease in spots (3 to *n*) corrected for material appearing in spot *F*, i.e. (ii) − (i)	(iii)	24		31		29		47		75		115		89	
Decrease in spot 1	(iv)	6		25		35		47		82		110		47	
Ratio (iii)/(iv)	(v)	4·00		1·24		0·83		1·00		0·92		1·05		0·53	
Sum of decreases in spot 1 and spots (3 to *n*) i.e. (iii) + (iv)	(vi)	30		56		64		94		157		225		136	
Increase in spot 2	(vii)	26		48		71		93		133		160		206	
Ratio (vi)/(vii) (average of these values 1·07)	(viii)	1·15		1·17		0·90		1·01		1·18		1·41		0·66	

The left-hand* and right-hand† columns for each concentration of added sucrose refer to values before and after incubation, respectively.

the increase in spot 2, and with high concentrations of sucrose the absolute errors in measuring the ketose contents of both spots 1 and 3 to n become considerable. Thus, an error of 5% in the estimation of spot 1 in the samples containing no added sucrose could cause an error in this ratio of about 55%, whilst a similar error in estimation of the sample containing the highest level of added sucrose could cause an error of about 100%.

If the results with these two samples are ignored, the average value of the ratio $\frac{\text{decrease in spots 3 to } n}{\text{decrease in spot 1}}$ is 1·01.

These results confirmed the previous assumption that the material appearing as spot 2 was derived both from sucrose and from the polysaccharide material contained in spots 4 to n (Table 9*b*, vi, vii and viii). A hypothesis in accordance with the facts presented is that fructose residues are transferred from polysaccharide to sucrose molecules, forming a trisaccharide in the first instance:

$$(\text{fructose})_n + \text{glucose-fructose} \rightarrow (\text{fructose})_{n-1} + \text{glucose-(fructose)}_2.$$

Further fructose residues might then be transferred to the trisaccharide, yielding a tetrasaccharide, and so on.

Action on mixtures of inulin and sucrose

Preparation of active concentrates. According to the above hypothesis, systems not containing a fructose donor (i.e. polysaccharide) would not show the reaction, which would explain why no significant amounts of oligosaccharide had been produced when ammonium sulphate precipitates were incubated with sucrose alone. The traces sometimes formed under such conditions may have been due to the presence of a small amount of undialysable polysaccharide from the original extract.

Confirmatory evidence in support of the hypothesis was obtained by an experiment in which a mixture of sucrose and inulin was incubated with an ammonium sulphate fraction (prepared between 0·25 and 0·55 saturation). Spot 2 was formed; when the enzyme preparation was incubated with inulin alone, or sucrose alone, it was not (see Fig. 2).

The preparation used in this experiment had been concentrated with respect to its hydrolytic activity (see Edelman & Bacon, 1951), and its transferring activity was fairly weak. It was found that addition of ammonium sulphate to 0·60 saturation in fresh extracts precipitated material which, when taken up in a small volume of water and dialysed, showed rather higher activity as judged qualitatively in systems containing inulin and sucrose. In later experiments spots 2 and 3 and traces of higher spots were produced in such systems. Although the reaction was usually carried out in the presence of about 0·02M-phosphate buffer (pH 6·5) inorganic phosphate did not appear to be necessary for the production of the higher spots. Similar systems set up in the presence and absence of phosphate buffer showed no difference in activity as judged qualitatively.

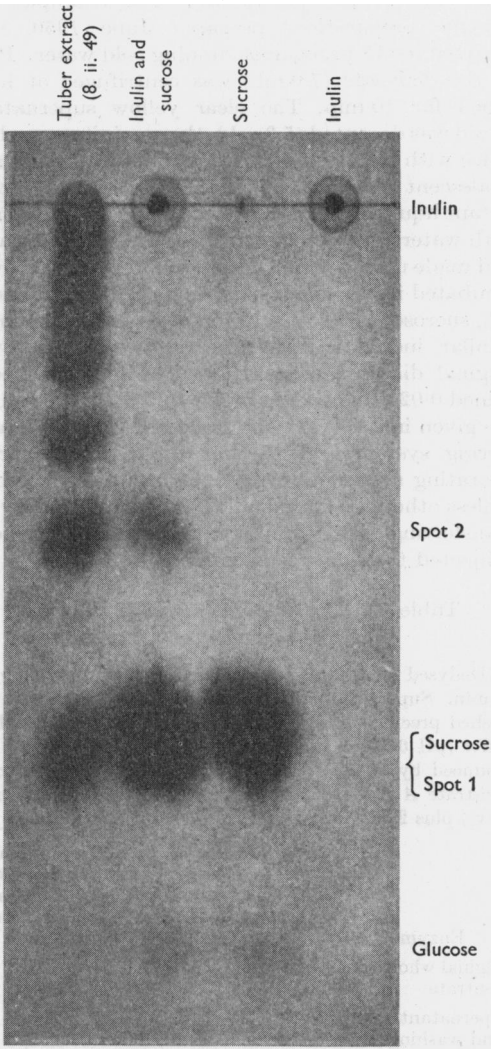

Fig. 2. Enzyme preparation, made February 1949, incubated with 2·5% added inulin, with 5% added sucrose, and with both, for 24 hr. at 30° in 0·1M-phosphate buffer (pH 6·5). Chromatogram run in butanol-acetic 2 days; sprayed with benzidine-trichloroacetic.

The same reaction was found to occur with a 0·60 saturation with ammonium sulphate precipitate of an aqueous extract of Dahlia tubers.

Monosaccharide liberation. The enzyme preparations just described were from extracts made towards the end of the storage season (end of May–June) and were found to liberate free glucose and fructose from

sucrose. Such dialysed preparations on centrifugation at high speed (i.e. about 10,000 *g*) gave a copious precipitate of material which had not redissolved during dialysis. The clear supernatant liquid contained the transferring activity but very little sucrose-hydrolysing activity, most of which was contained in the precipitate. For example: an enzyme preparation, prepared June 1950, was dialysed for 18 hr. against running cold water. Part of the dialysate (7·0 ml.) was centrifuged at high speed for 10 min. The clear yellow supernatant liquid was decanted (5·3 ml.), the precipitate washed twice with water by centrifugation and the slightly opalescent washings added to the original supernatant liquid. This solution was made up to 10·6 ml. with water. The precipitate was suspended in water and made up to 7·0 ml. These two preparations were incubated in a solution containing 2·5 % inulin and 5 % sucrose, and in a solution of 5 % sucrose alone. Similar incubations were carried out using the original dialysed preparation. The incubates contained 0·02 M-phosphate buffer (pH 6·5). The results are given in Table 10, and indicated that the transferring system and part of the monosaccharide liberating system were independent of each other. Unless otherwise stated, all the preparations having transferring activity referred to below had been subjected to high-speed centrifugation.

Table 10. *Distribution of transferring and fructose-liberating activities*

(Dialysed concentrate centrifuged at about 10,000 *g* for 10 min. Supernatant liquid combined with washings, and washed precipitate, tested. Incubated in 0·02 M-phosphate buffer (pH 6·5) for 18 hr. at 30°. Values expressed as mg. produced by the equivalent of 10 ml. original concentrate. Substrate *A*: 5 % sucrose (w/v); substrate *B*: 5 % sucrose (w/v/) plus 2·5 % inulin (w/v).)

Enzyme	Liberation of free fructose: Substrate *A*	Liberation of free fructose: Substrate *B*	Ketose content of spots formed with R_F < sucrose > inulin from substrate
Original whole concentrate	108	177	221
Supernatant liquid and washings	29	39	227
Precipitate	105	131	52·5

Effect of varying concentrations of sucrose and inulin. Variations in the concentrations of sucrose and inulin had a considerable effect upon the transferring activity at the levels tested (enzyme preparation not centrifuged); see Table 11. The free fructose liberated by the hydrolytic system was not affected by variation in sucrose concentration.

Degree of concentration of enzymic activity. In Table 6 the increase in spot 2 during 6 hr. incubation with 4 ml. fresh extract was 81 μg. ketose/20 μl. incubate; after 6 hr. incubation with 1 ml. enzyme concentrate it was 123 μg. The degree of concentration of activity was thus (4 × 123):81 or about 6:1. A calculation based on the figure for fresh extract with 5% sucrose in Table 9 and that for the concentrate with 2% inulin and 5% sucrose in Table 11 shows no appreciable concentration, but in this case the comparison may not be valid, since there was four times as much fructose in carbohydrates other than sucrose in the former incubation.

Table 11. *Effect of varying concentrations of inulin and sucrose on the activity of an enzyme concentrate*

(Ammonium sulphate concentrate (overall dilution × 5); incubated 18 hr. at 30° in about 0·01 M-phosphate buffer (pH 6·5) with various concentrations of inulin and sucrose. Values expressed as μg. ketose/20 μl. incubate.)

Inulin (%)	Sucrose (%): 1·0	3·0	5·0
	(*a*) Formation of spot 2		
0·5	13	20	24
2·0	19	40	55
	(*b*) Formation of spot *F*		
0·5	55	57	49
2·0	143	157	151

Isolation of spot 2 formed from inulin and sucrose. An enzyme preparation (10 ml.) was incubated at 30° in a total volume of 30 ml., containing 1·5 g. sucrose, 1·5 g. inulin, and 0·03 M-phosphate buffer (pH 6·5). After 20 hr. a slight turbidity was seen, presumably due to the precipitation of some of the inulin. After 24 hr. 2 vol. of ethanol were added, and the mixture filtered after 30 min. The precipitate was washed with 65 % ethanol and the filtrate and washings evaporated to dryness under reduced pressure with a water-bath temperature of 40°, yielding 1·75 g. of yellow syrup. 1·58 g. of this were placed on a column of 75 g. Whatman Ashless Tablet cellulose powder, 35 cm. high and 2·8 cm. in diameter, and the chromatogram developed with *n*-butanol-N-aqueous ammonia mixture (Hough, Jones & Wadman, 1949). Examination of the effluent by paper partition chromatography showed that sucrose was present between 1180 and 3015 ml. of total effluent, the last 755 ml. of this fraction containing also spot 2. The fraction between 3015 and 4600 ml. contained only spot 2 and after 4600 ml. both spots 2 and 3.

Part of the effluent containing only spot 2 (855 ml.) was taken to dryness as before, the syrup taken up in 50 % ethanol and treated with charcoal. When again taken to dryness it consisted of 165 mg. of slightly yellow syrup, which could not be induced to crystallize. It was analysed without fractionation (see Table 12). The remainder of the spot 2 fraction (in aqueous butanol) was reduced to a volume of

about 20 ml., when the precipitate that appeared was separated by centrifugation, washed with *n*-butanol and absolute ethanol, dried *in vacuo*, and analysed (Table 12); this material would appear to have been relatively free from the inert material which contaminates the effluent. The ratio of fructose:glucose of about 2 : 1 calculated from these analyses supports the view that spot 2 is a trisaccharide formed by the addition of a fructose residue to sucrose.

Table 12. *Analyses of spot 2 formed from inulin and sucrose*

(All weights are expressed as mg.)

		Unfractionated material from column	Residue after extraction with *n*-butanol
Dry weight of syrup	(i)	137	14·6
RS before acid hydrolysis	(ii)	8·4	0·32
RS after acid hydrolysis*	(iii)	92·5	13·4
Total ketose	(iv)	58·5	8·7
Total RS as percentage dry weight from (i) and (iii)		67·5	92
Free RS as percentage total RS from (ii) and (iii)		9·1	2·4
Ratio fructose : glucose from (iii) and (iv)		1·7 : 1	1·9 : 1
Ratio fructose : glucose from (iii) – (ii) and (iv)		2·3 : 1	2·0 : 1

* Corrected from fructose standard, assuming one-third glucose.

Specificity of the enzymic action

The replacement of inulin in the system by ethanol fractions of the tuber carbohydrates. The possibility that various ethanol fractions of the tuber carbohydrates might give a greater effect than inulin was first investigated. Fractions precipitated between 0–80, 70–80 and 80–90 % ethanol, were prepared by adding the appropriate volume of ethanol to fresh extract, prepared April 1950, and allowing the solutions to stand for 2 hr. at room temperature. The supernatant liquid was decanted and the sticky precipitates (a syrup in the case of the 80–90 % fraction) well drained; the 0–80 % fraction was reprecipitated twice from 80 % aqueous ethanol. The fractions were taken up in water. Both the 70–80 and 80–90 % fractions contained considerable amounts of carbohydrate with R_F greater than zero, there being very little material with zero R_F in the latter case. The 0–80 % fraction contained no material with R_F appreciably greater than zero. Qualitative chromatographic comparison of incubates after 15 hr. at 30° in 0·02 M-phosphate buffer (pH 6·5), containing these fractions (at 5 % concentration of combined ketose) together with sucrose (5 %) and an enzyme preparation (1 vol. in 3; prepared May 1950), showed a somewhat greater increase of spot 2 in the case of the 70–80 and 80–90 % fractions as compared with a similar system using inulin. The 0–80 % fraction, however, showed a rather lower activity than inulin. There being no very striking differences between these systems and those containing inulin, it was not considered profitable to use ethanol fractions routinely as fructose donors in place of inulin, because the 70–80 and 80–90 % fractions were considerably contaminated with spots 2 and 3, etc., and because inulin is a more definitely characterized material than any obtained by precipitation between arbitrarily defined limits of ethanol concentration (cf. Dean, 1904).

Replacement of sucrose by hexose. Analogous reactions involving glucose or fructose would be expected to give, in the first instance, disaccharide.

An enzyme preparation, prepared June 1950, showed no formation of intermediate carbohydrate material when incubated with a mixture of glucose and inulin. With a mixture of fructose and inulin, however, spots were formed, each of which, although very similar in position to spots 1, 2 or 3 on butanol-acetic acid chromatograms, had a somewhat greater R_F than the corresponding tuber component (see Fig. 3). Similar results were obtained when glucose was present in addition to fructose and inulin. Control incubations of enzyme with glucose and/or fructose in the absence of inulin, and inulin in the absence of hexose, showed no such results.

The compounds formed by the reaction between inulin and fructose resembled spots 1, 2, etc., in being attacked by invertase, but differed in containing no combined glucose. Thus their reaction with benzidine-trichloroacetic was typical of fructose in giving a weak yellow instead of the strong chocolate-brown colour typical of glucose; the spots showed strongly with the phloroglucinol reagent. Conclusive evidence for the absence of combined glucose was obtained by two-dimensional chromatography, invertase being used to hydrolyse the spots after the first development; this hydrolysis gave rise to fructose alone.

When treated with an acetone solution of silver nitrate, and then with ethanolic sodium hydroxide as described by Trevelyan, Procter & Harrison (1950) the spots showed a much slower reducing action than fructose; sucrose and raffinose spots on the same chromatogram reacted at the same time although less intensely. This could not be taken as evidence that they were non-reducing, since turanose also reacted almost as slowly as sucrose.

The di- and trisaccharide appearing during the early action of hydrolytic enzyme preparations on inulin (Edelman & Bacon, 1951) behaved similarly with respect to all these tests and are thus probably due to a reaction between the free fructose formed from inulin, and unchanged inulin.

Replacement of sucrose by other oligosaccharides. Using 1 vol. of an uncentrifuged enzyme concentrate

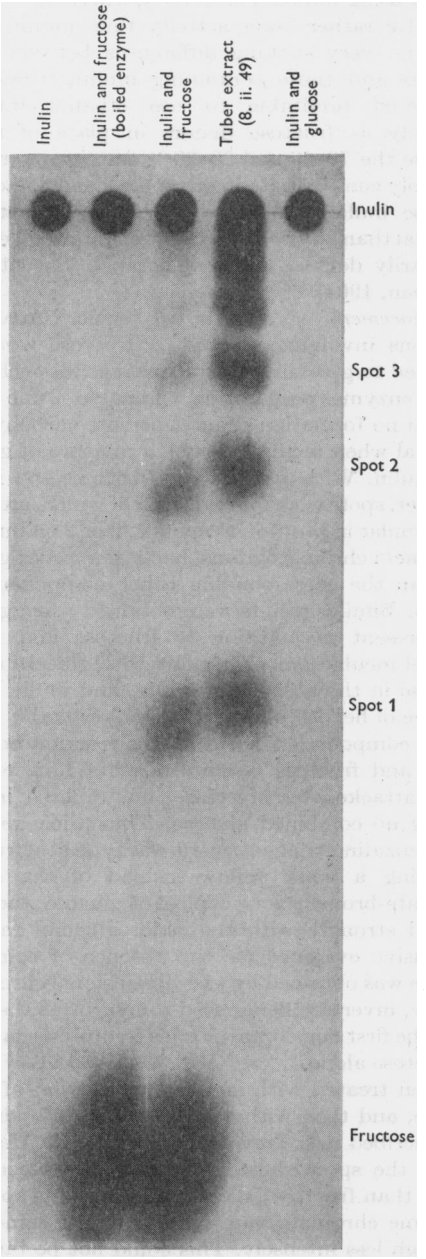

Fig. 3. Enzyme preparation, made June 1950, incubated with various combinations of 5% added inulin, 7·5% added fructose and 7·5% added glucose for 41 hr. at 30° in 0·1M-phosphate buffer (pH 6·5). Chromatogram run in butanol-acetic for 2 days; sprayed with phloroglucinol.

in 3 with inulin (5%) as fructose donor, lactose, maltose and trehalose (all 5%) showed chromatographically no differences after incubation (up to 30 hr.) from control incubations with these sugars in the absence of inulin. The incubations were carried out in 0·02M-phosphate buffer (pH 6·5). There was some lactase and maltase activity and a considerable trehalase activity as judged qualitatively.

However, in a similar experiment using raffinose (7·5%), there appeared after 5 hr. a spot with R_F smaller than that of raffinose, and with approximately the same intensity as spot 2 formed during a parallel incubation with sucrose of about the same molar concentration (0·15M, i.e. 5%, w/v). On continued incubation (30 hr.) a further spot, with still smaller R_F, appeared. (Enzyme preparations after centrifugation still contained some melibiase activity, although the invertase activity had been eliminated.)

Experiments with melezitose (kindly supplied by Dr C. S. Hudson of Bethesda, Maryland, U.S.A.) also gave positive results. Incubates containing enzyme preparation (1 vol. in 3), 5% inulin and 5% melezitose in 0·02M-phosphate buffer (pH 6·5) showed, after 18 hr. at 30°, a spot with R_F smaller than that of melezitose on chromatograms run in butanol-acetic or phenol. Incubation in the absence of inulin showed no such spot; the melezitose was not hydrolysed.

Replacement of inulin by other polysaccharides. Various polysaccharides were incubated with the enzyme (uncentrifuged; 1 vol. in 3) and sucrose (5%). The incubations were carried out in 0·02M-phosphate buffer (pH 6·5).

Spots appeared with R_F smaller than that of sucrose with both starch and glycogen, but these spots also appeared in the controls incubated without sucrose. In the case of glycogen the spot immediately above sucrose (presumably maltose) was stronger in the control, and there was a series of weaker spots extending as a streak to the starting line both in the presence and absence of sucrose. These spots (reducing dextrins?) all gave the reddish colour typical of maltose with the benzidine-trichloroacetic spraying reagent. Small amounts of glucose were liberated from both starch and glycogen.

A grass levan (kindly supplied by Dr D. J. Bell) showed no spots other than those of the carbohydrates added, either in the presence or absence of sucrose. Irisin 'B' (dialysed before use) showed a trace of a spot in position '2' after 30 hr. incubation in the presence of sucrose. The spot did not appear in the absence of sucrose.

DISCUSSION

The experiments described show the presence in extracts of artichoke tubers of an enzyme system which catalyses the transference of fructose residues from inulin and associated polysaccharides to sucrose. No evidence, however, is available to show

how small the polysaccharide donor or how large the oligosaccharide acceptor may be; it is not excluded that relatively small molecules may take either part in the reaction, although sucrose does not donate fructose to sucrose to any appreciable extent.

The latter observation calls attention to the similarities in specificity (as far as this has been tested) between the hydrolytic enzyme system described previously (Edelman & Bacon, 1951) and the donor side of the transfructosidation system. The specificity of the acceptor side of the transfer reaction is low, requiring only a fructose residue, free or combined. (One acceptor, melezitose, is not attacked hydrolytically by the enzyme preparation studied.)

The question therefore arises whether the same enzyme is responsible for both the hydrolytic and the group-transfer reactions. Such a view might receive support from recent observations on other enzymes attacking hydrolysable substrates. For example, Rabaté (1935, 1937) described transglycosidation in plant extracts which were capable of hydrolysing certain heterosides; Axelrod (1948*a*, *b*) and Appleyard (1948) described transphosphorylation catalysed by acid phosphatase preparations; and Fruton (1950; Johnston, Mycek & Fruton, 1950) described a transpeptidation reaction with cysteine-activated papain. Fruton (1950) suggested a mechanism to account for his observations, and remarked 'it will be important to examine the possibility that some of the enzymes considered to function solely as catalysts in hydrolytic reactions may have an important physiological role in the catalysis of replacement reactions' (Johnston *et al.* 1950). This view was further borne out by the observation of Meyerhof & Green (1950) that alkaline phosphatase would catalyse transphosphorylation reactions, and those of Blanchard & Albon (1950) and Bacon & Edelman (1950) that yeast invertase preparations formed substances presumed to be trisaccharides during their hydrolytic action on sucrose.

If one assumes that such dual activities are the property of a single enzyme it may be postulated that the primary action of the enzyme is to combine with the substrate, which may be represented as *A*-*B*. Three (or perhaps more) possibilities then present themselves: (1) that the elements of water may combine with the substrate, so that hydrolysis takes place, (2) that *B* (and less probably *A* as well) may be exchanged for a substance of similar structure (*C*) yielding the compound *A*-*C*, (3) that *B* may be exchanged with a phosphate radical, the substance *A*-phosphate being formed.

In the case of acid phosphatases only the alcohol or phenol moiety is exchangeable, and water may take its place. With sucrose phosphorylase only the fructose residue is exchangeable, either by other sugars (L-sorbose, D-xyloketose, L-arabinose), or by phosphate, but not by water (cf. Hassid & Doudoroff, 1950). All three reactions may be regarded equally as group-transfer reactions: to water, to an organic acceptor, or to phosphate, respectively.

Wholly hydrolytic enzymes and wholly transferring enzymes would on this assumption represent merely the extremes of a general type. In the case of certain enzymes that attack sucrose one may discern a gradation extending from yeast invertase, which shows relatively slight transferring activity, through the mould invertases, which show appreciable transfer (Edelman, Bacon & Bealing, 1950), to levansucrase (Hestrin *et al.* 1943; Hestrin & Avineri-Shapiro, 1944) which produces a polysaccharide and only very little free fructose.

Changes in pH might alter the ratio of the hydrolytic to group-transfer activities even if these were the property of a single enzyme. Thus Johnston *et al.* (1950) found that at a higher pH transpeptidation was increased relative to hydrolysis. Axelrod (1948*a*), however, found little difference in the pH optima for the two activities of his orange juice phosphatase preparation. In artichoke tuber extract the pH optimum of transfructosidation is higher than that for hydrolysis of the tuber carbohydrates.

A consequence of (1) and (2) is that there should be competition between water and the other acceptors in the system. Since the concentration of water is already at its maximum in an aqueous medium, increase in the absolute concentration of another acceptor would mean an increase in its concentration relative to that of water. This is consistent with the increase in transfructosidation with increasing sucrose concentration shown in Table 9, and might explain the high molar concentrations apparently needed for maximum transfer, which has not so far been achieved in our experiments.

Whether the rate of hydrolysis should alter in the presence of acceptors other than water cannot be predicted. It is conceivable that hydrolysis might be reduced by the presence of an acceptor (cf. orange juice phosphatase/methanol/methyl phosphate system, Axelrod, 1948*a*; also Rabaté, 1937) or increased if the product of group-transfer were more readily hydrolysed (cf. prostatic acid phosphatase/*iso*propanol/phenolphthaleinphosphate system, Appleyard, 1948). The latter possibility recalls the earlier observations of Behrens & Bergmann (1939) on the 'co-substrate' effect of certain peptides with proteolytic enzymes. The fact that the liberation of free fructose does not alter much with a fivefold increase in acceptor concentration and a tenfold increase in the rate of transfructosidation thus cannot be taken as evidence that the two systems are independent.

From these considerations it follows that the question of the identity of the hydrolytic and

fructose-transferring enzyme system of the artichoke tuber remains an open one. However, the existence of a transfructosidation reaction lends further support to the view that the structures of all the components of the tuber carbohydrates are based upon that of sucrose (Bacon & Edelman, 1951; cf. also Bell, 1947).

SUMMARY

1. From artichoke tubers enzyme preparations have been obtained which catalyse the transference of fructose residues from inulin and associated polysaccharides to sucrose, thereby forming a trisaccharide and higher oligosaccharides.

2. Evidence has also been obtained for the transference of fructose residues to raffinose, melezitose, and to free fructose, but not to maltose, lactose, trehalose or glucose.

3. The liberation of free hexose which took place in all the preparations examined does not appear to be connected quantitatively with the group-transfer reaction.

4. The reaction, for which the name 'transfructosidation' is proposed, is discussed in the light of existing knowledge of hydrolytic and group-transferring enzyme systems.

Note added 22 *June* 1951. Since this paper was submitted our attention has been drawn to the work of Dedonder (1950, 1951), from which he drew independently conclusions similar to our own about the nature of the artichoke fructosans, and suggested that phosphate was necessary for their synthesis from sucrose.

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REFERENCES

Appleyard, J. (1948). *Biochem. J.* **42**, 596.
Axelrod, B. (1948*a*). *J. biol. Chem.* **172**, 1.
Axelrod, B. (1948*b*). *J. biol. Chem.* **176**, 295.
Bacon, J. S. D. & Edelman, J. (1950). *Arch. Biochem.* **28**, 467.
Bacon, J. S. D. & Edelman, J. (1951). *Biochem. J.* **48**, 114.
Behrens, O. K. & Bergmann, M. (1939). *J. biol. Chem.* **129**, 587.
Bell, D. J. (1947). *Rep. Progr. Chem.* **44**, 222.
Blanchard, P. H. & Albon, N. (1950). *Arch. Biochem.* **29**, 220.
Dean, A. L. (1904). *Amer. chem. J.* **32**, 69.
Dedonder, R. (1950). *C.R. Acad. Sci., Paris*, **230**, 549, 997.
Dedonder, R. (1951). *C.R. Acad. Sci., Paris*, **231**, 790, 1134.
Edelman, J. & Bacon, J. S. D. (1950). *Biochem. J.* **47**, xlii.
Edelman, J. & Bacon, J. S. D. (1951). *Biochem. J.* **49**, 446.
Edelman, J., Bacon, J. S. D. & Bealing, F. J. (1950). Unpublished observations.
Fruton, J. S. (1950). *Yale J. Biol. Med.* **22**, 263.
Hassid, W. Z. & Doudoroff, M. (1950). *Advanc. Enzymol.* **10**, 123.
Hestrin, S. & Avineri-Shapiro, S. (1944). *Biochem. J.* **38**, 2.
Hestrin, S., Avineri-Shapiro, S. & Aschner, M. (1943). *Biochem. J.* **37**, 450.
Hough, L., Jones, J. K. N. & Wadman, W. H. (1949). *J. chem. Soc.* p. 2511.
Johnston, R. B., Mycek, M. J. & Fruton, J. S. (1950). *J. biol. Chem.* **185**, 629.
Meyerhof, O. & Green, H. (1950). *J. biol. Chem.* **183**, 377.
Rabaté, J. (1935). *Bull. Soc. Chim. biol., Paris*, **17**, 572.
Rabaté, J. (1937). *C.R. Acad. Sci., Paris*, **204**, 153.
Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.

The Construction of a Counter-current Apparatus

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The introduction of an apparatus for counter-current extraction by Craig (1944) was a notable contribution to the separation of a wide variety of compounds. Recently Craig (1950) has devised a glass apparatus of large capacity.

In applying the counter-current principle to the separation of lipids it has been found necessary to design a new apparatus in which the fluid phases could easily be examined for emulsions and removed for centrifuging, if necessary. It was desirable that the instrument be suitable for both large and small volumes of fluid. These characteristics have been embodied in an apparatus of glass and plastic with 49 'theoretical plates'. Its simplicity, and low cost, would seem to adapt it to general laboratory use.

General description of apparatus

The apparatus (Figs. 1, 2) consists of an array of 50 pairs of vertical wide glass tubes with conical ends supported by 'Terry' clips on either side of a wooden frame. The lower ends of each pair are joined by narrow polythene tubing, and neighbouring pairs are joined at the upper ends by wide polythene tubing, the whole forming a continuous spiral around