

It is likely that a region containing a number of 'YG' repeats has been duplicated to give the previously defined larger 'A', 'B', 'C' and 'D' repeats (Giffard and Jacques, 1994). These larger repeats would thus contain a number of the smaller 'YG' motifs. Analysis of the 'YG' repeats was carried out to see if there was any evidence of a duplication event. A phylogenetic tree of the 16 'YG' repeats of GtfL was constructed using 'PHYLIP'. This analysis showed that some of the 'YG' repeat sequences were far more closely related to one another than other repeats of GtfL. These closely related repeats formed clusters on branches of the constructed phylogenetic tree (Fig. 6.4). The phylogenetic tree had three clusters which consisted of four or five repeating units. Closer analysis of these clusters suggested a triplet of sequential 'YG' repeats had been duplicated to give four series of these repeats. An alignment was constructed of the nucleotide sequence of the regions encoding the 'YG' repeats, L5-L7, L8-L10, L9-L12 and L13-L16 (the repeats were numbered L1 to L16 from the N-terminus of the C-terminal domain), with the region lying between L5 and L6 being omitted from the analysis as there was no similar spacing within the other triplets. This alignment produced 31% identity (Fig. 6.5A). This translates to 45% of the amino acids being identical or conserved within these triple 'YG' repeats, compared with only 5% if the 12 repeats are aligned as a group. Removing the first set of repeats, L5-L7, from the analysis increased the conserved or identical amino acids to 62% (Fig. 6.5B), suggesting the latter three sets were the result of more recent duplication and divergence events. Previous analysis of the GtfJ and GtfK nucleotide sequences also suggested duplication of a triplet of 'YG' repeats (Giffard and Jacques, 1994). It may be that the duplication and divergence events of the glucan-binding domain results in increased receptor binding, however it may also enable the enzymes to avoid immune responses (Giffard and Jacques, 1994).

Recent studies have looked for potential antigenic epitopes within the catalytic and glucan-binding domains which give rise to antisera able to inhibit GTF activity. A peptide construct derived from a major repeating unit of GtfB of *S. mutans* GS5 elicited antisera able to inhibit GTF-I and some GTF-S activity of *S. sobrinus* 6715 (Smith *et al.*, 1993). The authors suggest that use of these highly conserved regions of the GTFs may allow the development of a synthetic

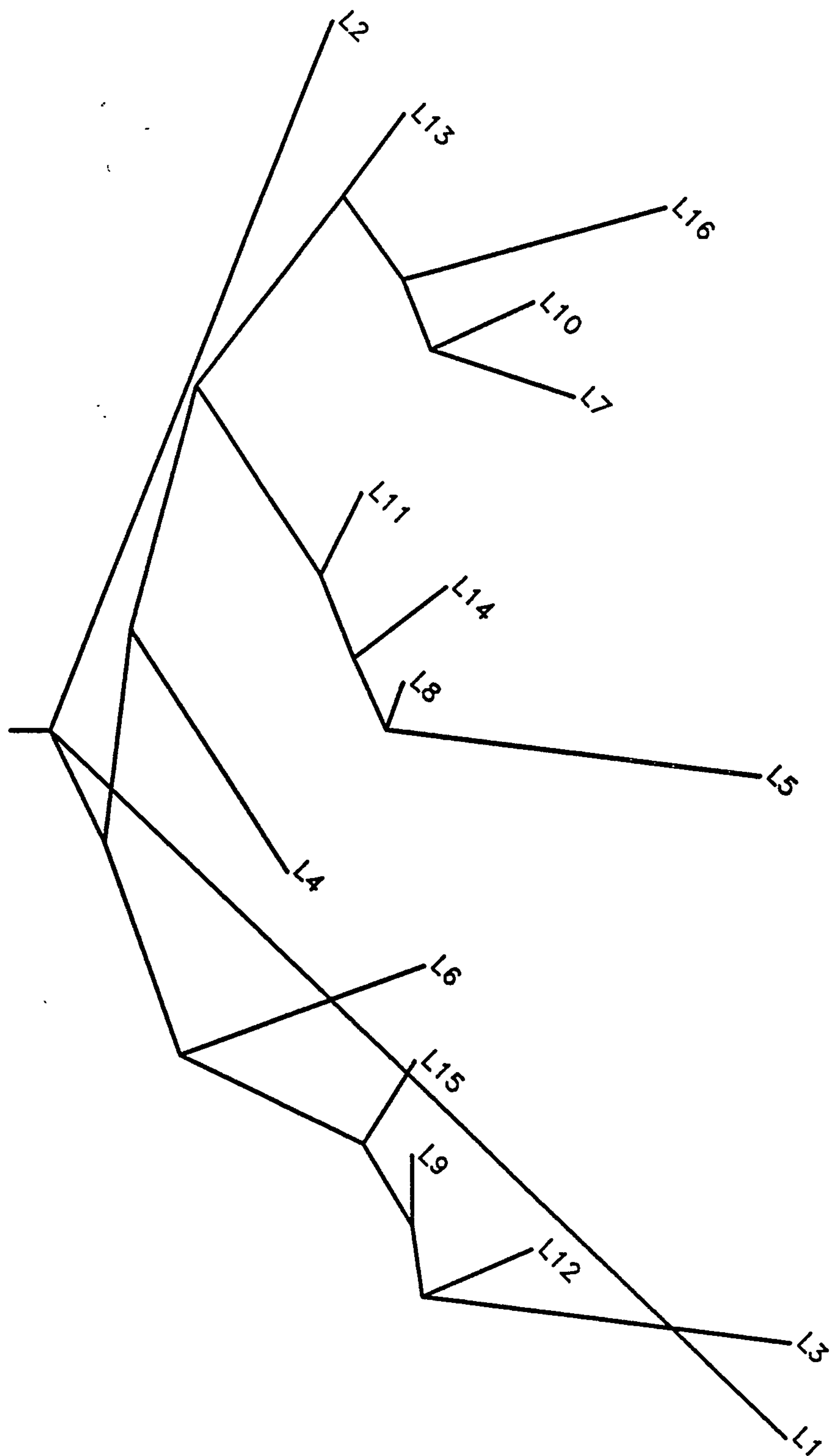


Fig. 6.4. Phylogenetic tree constructed from an alignment of the 16 'YG' repeats of GtfL.

Fig. 6.6. Homology between the in frame nucleotide sequence coding for the 'YG' repeats L5-7, L8-10, L11-13, and L14-16 (A); L8-10, L11-13 and L14-16 (B). The asterisks show positions of identity in the nucleotide sequence. The capital letters show the amino acids that are identical, while the lower case letters show the conserved amino acids in the aligned sequences.

peptide based vaccine. However, the duplication and divergence events that appear to be occurring in the glucan binding domain may be an ongoing event which may result in enough sequence variability to allow evasion of the immune system. Immunogens derived from the highly conserved catalytic region may therefore prove more effective in eliciting a long term immune response. While antisera raised against peptides from within the region of the catalytic domain containing the conserved aspartic acid residue of GTF-I and GTF-S of *S. sobrinus* (Section 1.12) were unable to inhibit GTF activity (Cope and Mooser, 1993), those antisera raised against regions preceding this active site amino acid (amino acids 342-356 GtfB; 435-453 GtfC) inhibited the synthesis of water-insoluble glucan and, in some cases, water-soluble glucan to a lesser degree (Dertzbaugh and Macrina, 1990; Chia *et al.*, 1993).

6.3.2 Analysis of *gtfM*

6.3.2.1 Nucleotide Sequence

The nucleotide sequence of *gtfM* was determined in both directions and is shown in figure 6.6. The *gtfM* gene is encoded by a 4730bp open reading frame with a putative ribosome-binding-site (GGATGA) 10bp upstream of the start codon. The potential promoter for this gene has a -10 (TATCATA) at base 53 and a -35 (TGTAATT) at base 37 (Fig. 6.6).

6.3.2.2 Deduced Amino Acid Sequence

The GtfM protein has a predicted molecular weight of 175 278 with a pI of 7.93. The cleavage site of the signal sequence of GtfM was predicted by the 'sigseq' program (6.3.1.2) to be after amino acid 38 (Fig. 6.6) and would result in a mature protein with a molecular weight of 171 169 and a pI of 6.77. pI values of GTFs are known to vary greatly and have been used to determine different activities in culture fluid (Russell *et al.*, 1987). The predicted pI of GtfM is much higher than that of the other Gtfs of *S. salivarius*. Interestingly, the pI of the mature form of GtfM is similar to that of the previously uncloned primer-independent GTF activity detected in the culture fluid of *S. salivarius* by isoelectric

Fig. 6.6. Nucleotide sequence and deduced amino-acid sequence of *gtfM*. A potential ribosome binding site (RBS), -10 and -35 promoter elements and a signal cleavage site (arrow) are indicated.

10	20	30	40	50	60
CAGAGATTTA	TGAAAAGAAG	ATGATTTTTT	CCTATTGTGA	ATTGTCTGA	ATATATCATA
			-35		-10
70	80	90	100	110	
GAGTAGAGAT	GACAAACAGAA	AAAAGGATGA	TTGATATAG	ATG GAA AAT AAG GTA CGT	
		RBS		Met Glu Asn Lys Val Arg	
				gtfM→	
120	130	140	150	160	
TTT AAA CTA CAC AAG GTT AAG AAA AAC TGG GTA ACT ATT GGG GTG ACC ACT					
Phe Lys Leu His Lys Val Lys Lys Asn Trp Val Thr Ile Gly Val Thr Thr					
170	180	190	200	210	
CTC TCA ATG GTT GCC TTG GCA GGT GGA AGC CTC CTA GCT CAA GGA AAA GTA					
Leu Ser Met Val Ala Leu Ala Gly Gly Ser Leu Leu Ala Gln Gly Lys Val					
					signal sequence cleavage site ↑
220	230	240	250	260	270
GAG GCC GAT GAG ACG AGC GCA CCT AAC GGT GAC GGC TTG CAG CAA CTG AGT					
Glu Ala Asp Glu Thr Ser Ala Pro Asn Gly Asp Gly Leu Gln Gln Leu Ser					
280	290	300	310	320	
GAG GAT GGG ACT GCC AGT CTA GTG ACG ACA ACA ACT GTT ACT GAG CAA GCT					
Glu Asp Gly Thr Ala Ser Leu Val Thr Thr Thr Thr Val Thr Glu Gln Ala					
330	340	350	360	370	
AGT GCT CAA GCA AGT GTG TCA GCA GTA GCA ACA GCC AGC GTA AGT CAC GAA					
Ser Ala Gln Ala Ser Val Ser Ala Val Ala Thr Ala Ser Val Ser His Glu					
380	390	400	410	420	
ACA AGC TTC CAG GCG GCG ACA AGT GCA GTC AGC CAG GAG GCA ACT GCT CAA					
Thr Ser Phe Gln Ala Ala Thr Ser Ala Val Ser Gln Glu Ala Thr Ala Gln					
430	440	450	460	470	
GCA CAA ACT AGT CCA GTT GCC AGT CAA GAA GTG GCA GTA TCT TCG CAA ACT					
Ala Gln Thr Ser Pro Val Ala Ser Gln Glu Val Ala Val Ser Ser Gln Thr					
480	490	500	510	520	
CAA TCC AGT GGC CAA GAG ACA CAG ACT ACT GAA CAG GTG TCA CAA GGT CAG					
Gln Ser Ser Gly Gln Glu Thr Gln Thr Thr Glu Gln Val Ser Gln Gly Gln					
530	540	550	560	570	
ACA TCA ACT CAA GTA GCT GGG CAA ACA AGT GCT CAG TCT ACT CCA AGT GTG					
Thr Ser Thr Gln Val Ala Gly Gln Thr Ser Ala Gln Ser Thr Pro Ser Val					
580	590	600	610	620	
ACA GAA CAA GCA AGA CCT AGA GTC TTG ACC AAT GCA GCG CCA GCA ATT GCC					
Thr Glu Gln Ala Arg Pro Arg Val Leu Thr Asn Ala Ala Pro Ala Ile Ala					
630	640	650	660	670	
ACA CGC GCT GCT GAT AGC ACT ATT CGT ATC AAT GCC AAC CGC AAT ACT AAC					
Thr Arg Ala Ala Asp Ser Thr Ile Arg Ile Asn Ala Asn Arg Asn Thr Asn					
680	690	700	710	720	
ATC ACG ATT ACG GCC AGC GGT ACG ACA CCA AAT GTA ACC ATT ATC ACA GGG					
Ile Thr Ile Thr Ala Ser Gly Thr Thr Pro Asn Val Thr Ile Ile Thr Gly					
730	740	750	760	770	780
CCA AAC ACG CCT AAA CCA AAC GTG ACG GTG ACA AGT CCA AAT GGC ACA AGA					
Pro Asn Thr Pro Lys Pro Asn Val Thr Val Thr Ser Pro Asn Gly Thr Arg					
790	800	810	820	830	
CCA AAT GTG ACC ATT GTA ACG CAG CCA AAT CAA CCC AAC AAA CCT GTT CAA					
Pro Asn Val Thr Ile Val Thr Gln Pro Asn Gln Pro Asn Lys Pro Val Gln					

	840		850		860		870		880							
CCA	AGT	CAA	CCG	TCT	CAA	CCT	AAC	AAG	CCG	GTC	CAA	CCA	AAT	CAG	CCA	AGT
Pro	Ser	Gln	Pro	Ser	Gln	Pro	Asn	Lys	Pro	Val	Gln	Pro	Asn	Gln	Pro	Ser
	890		900		910		920		930							
CTT	GAC	TAT	AAA	CCA	GTA	GCC	TCT	AAC	TTG	AAG	ACT	ATC	GAT	GGC	AAG	CAG
Leu	Asp	Tyr	Lys	Pro	Val	Ala	Ser	Asn	Leu	Lys	Thr	Ile	Asp	Gly	Lys	Gln
	940		950		960		970		980							
TAC	TAT	GTT	GAA	AAT	GGC	GTC	GTG	AAA	AAG	AAC	GCA	GCC	ATT	GAG	CTT	GAT
Tyr	Tyr	Val	Glu	Asn	Gly	Val	Val	Lys	Lys	Asn	Ala	Ala	Ile	Glu	Leu	Asp
	990		1000		1010		1020		1030							
GGG	CGT	CTT	TAT	TAT	TTC	GAT	GAG	ACT	GGA	GCT	ATG	GTG	GAT	CAA	AGT	AAA
Gly	Arg	Leu	Tyr	Tyr	Phe	Asp	Glu	Thr	Gly	Ala	Met	Val	Asp	Gln	Ser	Lys
	1040		1050		1060		1070		1080							
CCT	TTG	TAT	CGT	GCC	GAT	GCC	ATT	CCA	AAT	AAC	TCT	ATC	TAT	GCG	GTT	TAT
Pro	Leu	Tyr	Arg	Ala	Asp	Ala	Ile	Pro	Asn	Asn	Ser	Ile	Tyr	Ala	Val	Tyr
	1090		1100		1110		1120		1130							
AAC	CAG	GCC	TAT	GAT	ACG	TCA	AGT	AAG	AGT	TTT	GAA	CAC	TTG	GAT	AAT	TTC
Asn	Gln	Ala	Tyr	Asp	Thr	Ser	Ser	Lys	Ser	Phe	Glu	His	Leu	Asp	Asn	Phe
1140		1150		1160		1170		1180								
TTG	ACG	GCT	GAT	AGC	TGG	TAC	CGT	CCA	AAA	CAG	ATT	TTG	AAG	GAT	GGG	AAG
Leu	Thr	Ala	Asp	Ser	Trp	Tyr	Arg	Pro	Lys	Gln	Ile	Leu	Lys	Asp	Gly	Lys
1190		1200		1210		1220		1230								
AAT	TGG	ACA	GCT	TCA	ACT	GAG	AAA	GAT	TAT	CGT	CCA	CTT	TTG	ATG	ACT	TGG
Asn	Trp	Thr	Ala	Ser	Thr	Glu	Lys	Asp	Tyr	Arg	Pro	Leu	Leu	Met	Thr	Trp
1240		1250		1260		1270		1280		1290						
TGG	CCA	GAC	AAG	GTG	ACA	CAG	GTC	AAC	TAC	CTC	AAC	TAT	ATG	AGC	CAA	CAA
Trp	Pro	Asp	Lys	Val	Thr	Gln	Val	Asn	Tyr	Leu	Asn	Tyr	Met	Ser	Gln	Gln
	1300		1310		1320		1330		1340							
GGG	TTT	GGT	AAT	AAG	ACC	TAT	ACG	ACA	GAT	ATG	ATG	AGC	TAT	GAC	TTG	GCA
Gly	Phe	Gly	Asn	Lys	Thr	Tyr	Thr	Thr	Asp	Met	Met	Ser	Tyr	Asp	Leu	Ala
	1350		1360		1370		1380		1390							
GCT	GCT	GCC	GAA	ACG	GTT	CAA	CGA	GGC	ATC	GAG	GAA	CGT	ATT	GGT	CGT	GAG
Ala	Ala	Ala	Glu	Thr	Val	Gln	Arg	Gly	Ile	Glu	Glu	Arg	Ile	Gly	Arg	Glu
	1400		1410		1420		1430		1440							
GGC	AAT	ACC	ACT	TGG	CTT	CGC	CAG	TTG	ATG	TCA	GAC	TTC	ATC	AAA	ACA	CAG
Gly	Asn	Thr	Thr	Trp	Leu	Arg	Gln	Leu	Met	Ser	Asp	Phe	Ile	Lys	Thr	Gln
	1450		1460		1470		1480		1490							
CCT	GGC	TGG	AAC	TCT	GAG	AGT	GAG	GAC	AAT	CTC	TTA	GTT	GGT	AAA	GAC	CAC
Pro	Gly	Trp	Asn	Ser	Glu	Ser	Glu	Asp	Asn	Leu	Leu	Val	Gly	Lys	Asp	His
	1500		1510		1520		1530		1540							
TTG	CAA	GGA	GGC	GCT	CTG	ACC	TTC	CTA	AAT	AAT	AGT	GCG	ACA	AGT	CAT	GCC
Leu	Gln	Gly	Gly	Ala	Leu	Thr	Phe	Leu	Asn	Asn	Ser	Ala	Thr	Ser	His	Ala
	1550		1560		1570		1580		1590							
AAT	TCA	GAT	TTC	CGC	CTC	ATG	AAC	CGC	ACA	CCG	ACT	AAC	CAA	ACG	GGG	ACA
Asn	Ser	Asp	Phe	Arg	Leu	Met	Asn	Arg	Thr	Pro	Thr	Asn	Gln	Thr	Gly	Thr
	1600		1610		1620		1630		1640							
CGC	AAA	TAT	CAT	ATT	GAC	CGT	TCA	AAT	GGT	GGT	TAC	GAG	TTG	CTC	TTG	GCT
Arg	Lys	Tyr	His	Ile	Asp	Arg	Ser	Asn	Gly	Gly	Tyr	Glu	Leu	Leu	Leu	Ala
1650		1660		1670		1680		1690								
AAC	GAT	ATC	GAC	AAC	TCT	AAC	CCA	GCT	GTT	CAG	GCA	GAG	CAA	CTA	AAC	TGG
Asn	Asp	Ile	Asp	Asn	Ser	Asn	Pro	Ala	Val	Gln	Ala	Glu	Gln	Leu	Asn	Trp

1700	1710	1720	1730	1740	
CTC CAC TAC ATC ATG AAC ATT GGT TCT ATC CTT GGC AAT GAT CCA AGT GCC					
Leu His Tyr Ile Met Asn Ile Gly Ser Ile Leu Gly Asn Asp Pro Ser Ala					
1750	1760	1770	1780	1790	1800
AAC TTT GAC GGT GTT CGT ATC GAT GCG GTG GAC AAT GTG GAT GCG GAT CTC					
Asn Phe Asp Gly Val Arg Ile Asp Ala Val Asp Asn Val Asp Ala Asp Leu					
	1810	1820	1830	1840	1850
	TTG CAG ATT GCT TCT GAT TAC TTC AAG GAA AAA TAC CGT GTC GCA GAT AAT				
	Leu Gln Ile Ala Ser Asp Tyr Phe Lys Glu Lys Tyr Arg Val Ala Asp Asn				
	1860	1870	1880	1890	1900
	GAA GCA AAT GCC ATT GCT CAT TTG TCA ATC CTT GAA GCT TGG TCA TAC AAC				
	Glu Ala Asn Ala Ile Ala His Leu Ser Ile Leu Glu Ala Trp Ser Tyr Asn				
	1910	1920	1930	1940	1950
	GAC CAC CAA TAC AAC AAG GAT ACC AAG GGT GCT CAG TTG TCT ATC GAC AAT				
	Asp His Gln Tyr Asn Lys Asp Thr Lys Gly Ala Gln Leu Ser Ile Asp Asn				
	1960	1970	1980	1990	2000
	CCA CTA CGT GAA ACG CTT TTG ACG ACT TTC TTG CGT AAG AGC AAT TAC CGT				
	Pro Leu Arg Glu Thr Leu Leu Thr Thr Phe Leu Arg Lys Ser Asn Tyr Arg				
	2010	2020	2030	2040	2050
	GGC AGC TTG GAG CGC GTG ATT ACT AAC TCT CTT AAC AAT CGT TCA AGT GAG				
	Gly Ser Leu Glu Arg Val Ile Thr Asn Ser Leu Asn Asn Arg Ser Ser Glu				
	2060	2070	2080	2090	2100
	CAG AAA CAC ACG CCA CGT GAT GCC AAT TAT ATT TTC GTG CGA GCC CAT GAT				
	Gln Lys His Thr Pro Arg Asp Ala Asn Tyr Ile Phe Val Arg Ala His Asp				
	2110	2120	2130	2140	2150
	AGT GAA GTG CAA GCT GTT TTG GCT AAT ATC ATC AGC AAG CAG ATT AAT CCA				
	Ser Glu Val Gln Ala Val Leu Ala Asn Ile Ile Ser Lys Gln Ile Asn Pro				
2160	2170	2180	2190	2200	
AAA ACA GAT GGT TTC ACC TTC ACT ATG GAT GAG CTC AAA CAG GCC TTC GAA					
Lys Thr Asp Gly Phe Thr Phe Thr Met Asp Glu Leu Lys Gln Ala Phe Glu					
2210	2220	2230	2240	2250	
ATC TAC AAT GCG GAC ATC GCG AAG GCT GAT AAA AAG TAC ACC CAG TAC AAT					
Ile Tyr Asn Ala Asp Ile Ala Lys Ala Asp Lys Lys Tyr Thr Gln Tyr Asn					
2260	2270	2280	2290	2300	2310
ATC CCA GCT GCC TAT GCC ACA ATG TTG ACC AAC AAG GAT AGT ATC ACT CGT					
Ile Pro Ala Ala Tyr Ala Thr Met Leu Thr Asn Lys Asp Ser Ile Thr Arg					
	2320	2330	2340	2350	2360
	GTT TAC TAC GGG GAC CTC TTT ACC GAC GAT GGC CAA TAC ATG GCT GAA AAA				
	Val Tyr Tyr Gly Asp Leu Phe Thr Asp Asp Gly Gln Tyr Met Ala Glu Lys				
	2370	2380	2390	2400	2410
	TCA CCG TAC TAT AAT GCC ATC GAT GCC CTG CTC CGT GCT CGC ATC AAA TAT				
	Ser Pro Tyr Tyr Asn Ala Ile Asp Ala Leu Leu Arg Ala Arg Ile Lys Tyr				
	2420	2430	2440	2450	2460
	GTA GCA GGT GGT CAA GAC ATG AAG GTT ACT AAG CTT AAT GGC TAT GAA ATC				
	Val Ala Gly Gly Gln Asp Met Lys Val Thr Lys Leu Asn Gly Tyr Glu Ile				
	2470	2480	2490	2500	2510
	ATG TCA TCT GTG CGT TAT GGT AAG GGG GCA GAA GAA GCC AAC CAG CTT GGT				
	Met Ser Ser Val Arg Tyr Gly Lys Gly Ala Glu Glu Ala Asn Gln Leu Gly				
	2520	2530	2540	2550	2560
	ACT GCT GAA ACA CGC AAC CAA GGA ATG CTG GTC CTT ACA GCC AAC CGT CCA				
	Thr Ala Glu Thr Arg Asn Gln Gly Met Leu Val Leu Thr Ala Asn Arg Pro				

2570	2580	2590	2600	2610
GAT ATG AAG TTG GGA GCT AAT GAT CGT CTG GTA GTC AAT ATG GGA GCT GCC				
Asp Met Lys Leu Gly Ala Asn Asp Arg Leu Val Val Asn Met Gly Ala Ala				
2620	2630	2640	2650	2660
CAC AAA AAT CAG GCT TAC CGT CCA TTG CTT CTC AGC AAA TCG ACA GGT CTT				
His Lys Asn Gln Ala Tyr Arg Pro Leu Leu Leu Ser Lys Ser Thr Gly Leu				
2670	2680	2690	2700	2710
GCG ACC TAC CTC AAG GAT TCT GAT GTA CCA GCT GGA TTG GTT CGC TAT ACG				
Ala Thr Tyr Leu Lys Asp Ser Asp Val Pro Ala Gly Leu Val Arg Tyr Thr				
2720	2730	2740	2750	2760
GAC AAT CAA GGG AAC TTG ACC TTC ACG GCA GAT GAT ATT GCT GGT CAT TCA				
Asp Asn Gln Gly Asn Leu Thr Phe Thr Ala Asp Asp Ile Ala Gly His Ser				
2770	2780	2790	2800	2810
ACC GTT GAA GTT TCA GGT TAT TTG GCA GTT TGG GTG CCA GTG GGT GCC TCA				
Thr Val Glu Val Ser Gly Tyr Leu Ala Val Trp Val Pro Val Gly Ala Ser				
	2830	2840	2850	2860
	GAA AAC CAA GAT GCC CGA ACC AAG GCT TCG AGC ACC AAG AAG GGT GAG CAG			
	Glu Asn Gln Asp Ala Arg Thr Lys Ala Ser Ser Thr Lys Lys Gly Glu Gln			
	2880	2890	2900	2910
	GTC TTT GAA TCA TCA GCA GCT CTT GAT TCA CAA GTC ATT TAC GAA GGC TTC			
	Val Phe Glu Ser Ser Ala Ala Leu Asp Ser Gln Val Ile Tyr Glu Gly Phe			
	2930	2940	2950	2960
	TCA AAC TTC CAA GAT TTC GTT AAG ACA CCA AGT CAG TAC ACC AAC CGT GTT			
	Ser Asn Phe Gln Asp Phe Val Lys Thr Pro Ser Gln Tyr Thr Asn Arg Val			
	2980	2990	3000	3010
	ATT GCT CAA AAT GCC AAA CTC TTC AAA GAG TGG GGA ATC ACT TCC TTT GAA			
	Ile Ala Gln Asn Ala Lys Leu Phe Lys Glu Trp Gly Ile Thr Ser Phe Glu			
	3030	3040	3050	3060
	TTT GCG CCA CAG TAT GTG TCT AGC CAA GAC GGC ACT TTC TTG GAT TCT ATC			
	Phe Ala Pro Gln Tyr Val Ser Ser Gln Asp Gly Thr Phe Leu Asp Ser Ile			
	3080	3090	3100	3110
	ATT GAA AAT GGC TAC GCC TTC GAG GAC CGC TAC GAT ATT GCC ATG AGC AAG			
	Ile Glu Asn Gly Tyr Ala Phe Glu Asp Arg Tyr Asp Ile Ala Met Ser Lys			
	3130	3140	3150	3160
	AAC AAC AAA TAC GGT TCA CTC AAA GAC CTC ATG GAT GCC CTT CGT GCC CTT			
	Asn Asn Lys Tyr Gly Ser Leu Lys Asp Leu Met Asp Ala Leu Arg Ala Leu			
3180	3190	3200	3210	3220
CAC GCA GAA GGT ATC TCA GCC ATT GCT GAC TGG GTT CCA GAT CAA ATT TAC				
His Ala Glu Gly Ile Ser Ala Ile Ala Asp Trp Val Pro Asp Gln Ile Tyr				
3230	3240	3250	3260	3270
AAT CTC CCT GGA AAA GAA GTG GTA ACA GCT TCT CGT ACC AAT AGC TAC GGT				
Asn Leu Pro Gly Lys Glu Val Val Thr Ala Ser Arg Thr Asn Ser Tyr Gly				
3280	3290	3300	3310	3320
ACA CCA CGT CCA AAT GCT GAA ATC TAC AAT AGC CTC TAC GCA GCT AAA ACA				
Thr Pro Arg Pro Asn Ala Glu Ile Tyr Asn Ser Leu Tyr Ala Ala Lys Thr				
	3340	3350	3360	3370
	CGT ACC TTT GGA AAT GAC TTC CAA GGC AAG TAC GGT GGT GCC TTC CTT GAT			
	Arg Thr Phe Gly Asn Asp Phe Gln Gly Lys Tyr Gly Gly Ala Phe Leu Asp			
	3390	3400	3410	3420
	GAA TTG AAG GCA AAA TAC CCA GCA ATC TTT GAG CGC GTG CAG ATT TCA AAC			
	Glu Leu Lys Ala Lys Tyr Pro Ala Ile Phe Glu Arg Val Gln Ile Ser Asn			

3440	3450	3460	3470	3480
GGC CGT AAA TTG	ACT ACC AAT GAG	AAA ATC ACG CAA	TGG TCA GCC AAG	TAT
Gly Arg Lys Leu	Thr Thr Asn Glu	Lys Ile Thr Gln	Trp Ser Ala	Lys Tyr
3490	3500	3510	3520	3530
TTC AAT GGA AGC	AAT ATC CAA GGT	ACT GGA GCT CGC	TAT GTC CTA	CAA GAT
Phe Asn Gly Ser	Asn Ile Gln Gly	Thr Gly Ala Arg	Tyr Val Leu	Gln Asp
3540	3550	3560	3570	3580
AAC GCT ACC AAC	CAA TAC TTC AGC	GTC AAA GCA GGT	CAA ACC TTC	CTT CCT
Asn Ala Thr Asn	Gln Tyr Phe Ser	Val Lys Ala Gly	Gln Thr Phe	Leu Pro
3590	3600	3610	3620	3630
AAA CAA ATG ACT	GAA ATT ACT GGA	AGT GGT TTC CGT	AGG GTT GGA	GAT GAT
Lys Gln Met Thr	Glu Ile Thr Gly	Ser Gly Phe Arg	Arg Val Gly	Asp Asp
3640	3650	3660	3670	3680
GTC CAA TAC CTC	TCA ATT GGT GGC	TAC CTT GCT AAG	AAT ACC TTT	ATT CAA
Val Gln Tyr Leu	Ser Ile Gly Gly	Tyr Leu Ala Lys	Asn Thr Phe	Ile Gln
3690	3700	3710	3720	3730
GTC GGT GCC AAC	CAG TGG TAT TAC	TTT GAT AAG AAT	GGC AAC ATG	GTC ACA
Val Gly Ala Asn	Gln Trp Tyr Tyr	Phe Asp Lys Asn	Gly Asn Met	Val Thr
3740	3750	3760	3770	3780
GGT GAG CAG GTC	ATT GAT GGC AAG	AAA TAC TTC TTC	CTA GAC AAT	GGT CTC
Gly Glu Gln Val	Ile Asp Gly Lys	Lys Tyr Phe Phe	Leu Asp Asn	Gly Leu
3790	3800	3810	3820	3830
CAG CTA CGT CAT	GTC CTT CGC CAA	GGT AGT GAT GGT	CAT GTG TAT	TAT TAC
Gln Leu Arg His	Val Leu Arg Gln	Gly Ser Asp Gly	His Val Tyr	Tyr Tyr
3850	3860	3870	3880	3890
GAT CCT AAA GGG	GTT CAG GCC TTT	AAC GGA TTT TAT	GAT TTT GCG	GGT CCT
Asp Pro Lys Gly	Val Gln Ala Phe	Asn Gly Phe Tyr	Asp Phe Ala	Gly Pro
3900	3910	3920	3930	3940
CGC CAA GAC GTT	CGC TAC TTT GAT	GGC AAC GGT CAA	ATG TAT CGT	GGC CTC
Arg Gln Asp Val	Arg Tyr Phe Asp	Gly Asn Gly Gln	Met Tyr Arg	Gly Leu
3950	3960	3970	3980	3990
CAC GAC ATG TAT	GGC ACA ACC TTC	TAT TTT GAT GAA	AAG ACT GGT	ATT CAA
His Asp Met Tyr	Gly Thr Thr Phe	Tyr Phe Asp Glu	Lys Thr Gly	Ile Gln
4000	4010	4020	4030	4040
GCC AAA GAC AAG	TTC ATC CGT TTT	GCG GAC GGA CGC	ACG CGT TAC	TTC ATC
Ala Lys Asp Lys	Phe Ile Arg Phe	Ala Asp Gly Arg	Thr Arg Tyr	Phe Ile
4050	4060	4070	4080	4090
CCA GAT ACA GGA	AAT CTC GCA GTC	AAC CGA TTT GCG	CAA AAT CCT	GAG AAC
Pro Asp Thr Gly	Asn Leu Ala Val	Asn Arg Phe Ala	Gln Asn Pro	Glu Asn
4100	4110	4120	4130	4140
AAG GCT TGG TAT	TAC CTC GAT AGC	AAC GGT TAT GCC	GTG ACA GGA	CTA CAA
Lys Ala Trp Tyr	Tyr Leu Asp Ser	Asn Gly Tyr Ala	Val Thr Gly	Leu Gln
4150	4160	4170	4180	4190
ACC ATT AAC GGT	AAG CAG TAT TAC	TTT GAC AAT GAA	GGA CGT CAG	GTT AAG
Thr Ile Asn Gly	Lys Gln Tyr Tyr	Phe Asp Asn Glu	Gly Arg Gln	Val Lys
4200	4210	4220	4230	4240
GGA CAC TTT GTC	ACT ATC AAT AAC	CAA CGT TAC TTC	CTT GAT GGT	GAT AGT
Gly His Phe Val	Thr Ile Asn Asn	Gln Arg Tyr Phe	Leu Asp Gly	Asp Ser

4250	4260	4270	4280	4290	
GGT GAA ATT GCT CCG TCA CGC TTT GTG ACG GAA AAC AAC AAG TGG TAC TAT					
Gly Glu Ile Ala Pro Ser Arg Phe Val Thr Glu Asn Asn Lys Trp Tyr Tyr					
4300	4310	4320	4330	4340	4350
GTC GAT GGC AAT GGT AAA CTG GTT AAA GGT GCT CAG GTC ATC AAT GGT AAT					
Val Asp Gly Asn Gly Lys Leu Val Lys Gly Ala Gln Val Ile Asn Gly Asn					
	4360	4370	4380	4390	4400
CAC TAC TAT TTC AAC AAT GAT TAT AGC CAA GTC AAG GGT GCC TGG GCC AAC					
His Tyr Tyr Phe Asn Asn Asp Tyr Ser Gln Val Lys Gly Ala Trp Ala Asn					
	4410	4420	4430	4440	4450
GGC CGT TAC TAT GAT GGT GAC TCA GGT CAG GCC GTA AGC AAC CAA TTC ATT					
Gly Arg Tyr Tyr Asp Gly Asp Ser Gly Gln Ala Val Ser Asn Gln Phe Ile					
	4460	4470	4480	4490	4500
CAA ATT GCG GCT AAC CAA TGG GCT TAC CTT AAC CAA GAT GGT CAC AAA GTA					
Gln Ile Ala Ala Asn Gln Trp Ala Tyr Leu Asn Gln Asp Gly His Lys Val					
	4510	4520	4530	4540	4550
ACA GGT CTT CAA AAT ATT AAC AAT AAA GTT TAC TAT TTT GGT AGC AAT GGT					
Thr Gly Leu Gln Asn Ile Asn Asn Lys Val Tyr Tyr Phe Gly Ser Asn Gly					
	4560	4570	4580	4590	4600
GCT CAA GTC AAA GGT AAA TTG CTC ACT GTC CAA GGT AAG AAA TGT TAC TTT					
Ala Gln Val Lys Gly Lys Leu Leu Thr Val Gln Gly Lys Lys Cys Tyr Phe					
	4610	4620	4630	4640	4650
GAT GCC CAC ACA GGT GAG CAA GTG GTA AAC CGC TTT GTC GAA GCT GCA CGT					
Asp Ala His Thr Gly Glu Gln Val Val Asn Arg Phe Val Glu Ala Ala Arg					
	4660	4670	4680	4690	4700
GGC TGC TGG TAT TAC TTT AAC TCA GCT GGC CAA GCA GTG ACT GGA CAA CAG					
Gly Cys Trp Tyr Tyr Phe Asn Ser Ala Gly Gln Ala Val Thr Gly Gln Gln					
4710	4720	4730	4740	4750	
GTC ATC AAT GGT AAA CAA CTT TAC TTC GAC GGT TCA GGT CGT CAA GTT AAA					
Val Ile Asn Gly Lys Gln Leu Tyr Phe Asp Gly Ser Gly Arg Gln Val Lys					
4760	4770	4780	4790	4800	
GGA CGT TAT GTT TAT GTT GGT GGT AAA CGA CTC TTC TGC GAT GCC AAA ACT					
Gly Arg Tyr Val Tyr Val Gly Gly Lys Arg Leu Phe Cys Asp Ala Lys Thr					
4810	4820	4830	4840	4850	
GGT GAA TTG AGA CAG CGT CGC TAA TTAATAT GTACTTTAAA AAT					
Gly Glu Leu Arg Gln Arg Arg End					

focusing (Pitty *et al.*, 1989). There was, however, no similar band of activity for the GtfM expressed by the phagemid pGSG501 when subjected to isoelectric focusing gel analysis (Fig. 5.8). There was, however, a band of activity with a pI of 9.2, and another band of activity at a similar position those bands of GtfJ, GtfK and GtfL at pI 5.5.

The GtfM protein has already been noted as being relatively unstable as evidenced by a number of bands on SDS-PAGE (Fig. 5.7). Deletions up to 200 amino acids from the N-terminus of GtfM can be shown to result in proteins possessing increasing pI values, whereas similar deletions from the C-terminus lower the pI values (Table 6.1). However, none of these deletions gave a predicted pI as high as the 9.2 determined experimentally (Table 5.4).

Recombinant Gtfs are not secreted in *E. coli* (Aoki *et al.*, 1986; Hanada and Kuramitsu, 1988), and it is not known whether *S. salivarius* Gtfs are cleaved at the signal sequence cleavage site in this organism. Nonetheless, N-terminal analysis of the recombinant GtfI of *S. downei* expressed in *E. coli* indicated that cleavage of the signal sequence had occurred (Ferretti *et al.*, 1987). Furthermore, deletion analysis of the GtfB of *S. mutans* has suggested that while the signal sequence is functional in *E. coli*, it is the C-terminus that prevents secretion of the enzyme into the periplasm (Kato and Kuramitsu, 1991). It is reasonable to surmise that the *S. salivarius* Gtfs are processed in a similar manner in *E. coli* due to their high sequence similarity. Consequently, it is reasonable to suggest that it is post-translational processing that is responsible for the difference between the predicted and actual pI values of the recombinant GtfM.

6.3.2.3 Repeat Units of GtfM

GtfM has 21 'YG' repeats in its C-terminus (Fig. 6.7). Unlike the other Gtfs of *S. salivarius*, no evidence could be found for duplication of regions within the glucan-binding domain of this enzyme (Fig. 6.8). This may suggest that duplication events in this protein occurred earlier than in the other *S. salivarius* GTFs and that the repeat region has diverged to a greater extent.

Table 6.1. Effect of deletions from the N-terminus or C-terminus of GtfM on the predicted pI.

Deletion (amino acids)	pI of Predicted Protein when Deleted From:	
	N-terminus	C-terminus
50	7.2	6.6
100	8.2	6.3
150	8.6	6.2
200	8.3*	6.3

* further N-terminal deletions reduced the pI value.

M1	1146	NATNQYFSVKAGQTFLPKQMT	1166
M2	1176	VGDDVQYLSIGGYLAKNTFIQ	1196
M3	1198	GANQWYYFDKNGNMVTGEQVI	1218
M4	1218	IDGKKYFFLDNGLQLRHVLRQ	1238
M5	1240	SDGHVYYYDPKGVGAFNGFYD	1260
M6	1252	VQAFNGFYDFAGPRQDVRYFD	1272
M7	1264	PRQDVRYFDGNGQMYRGLHDM	1284
M8	1285	YGTTFYFDEKTGIQAKDKFIR	1305
M9	1308	DGRTRYFIPDTGNLAVNRFAQ	1328
M10	1331	ENKAWYYLDSNGYAVTGLQTI	1351
M11	1351	INGKQYYFDNEGRQVKGHFVT	1371
M12	1373	NNQRYFLDGDSGEIAFSRFVT	1393
M13	1394	ENNKWYYVDGNGKLVKGAQVI	1414
M14	1419	YYFNNDYSQVKGAWANGRYD	1439
M15	1432	WANGRYYDGD SGQAVSNQFIQ	1452
M16	1454	AANQWAYLNQDGHKVTGLQNI	1474
M17	1474	INNKVYYFGSNGAQVKGKLLT	1494
M18	1496	QGKKCYFDAHTGEQV VNR FVE	1516
M19	1518	ARGCWYYFNSAGQAVTGQQVI	1538
M20	1538	INGKQLYFDGSGRQVKGRYVY	1558
M21	1550	RQVKGRYVYVGGKRLFCDAKT	1570

Fig. 6.7. ‘YG’ repeat sequences from the C-terminal domain of GtfM. Numbers at the left and right of repeating units indicate the first and last amino acid of each repeat.

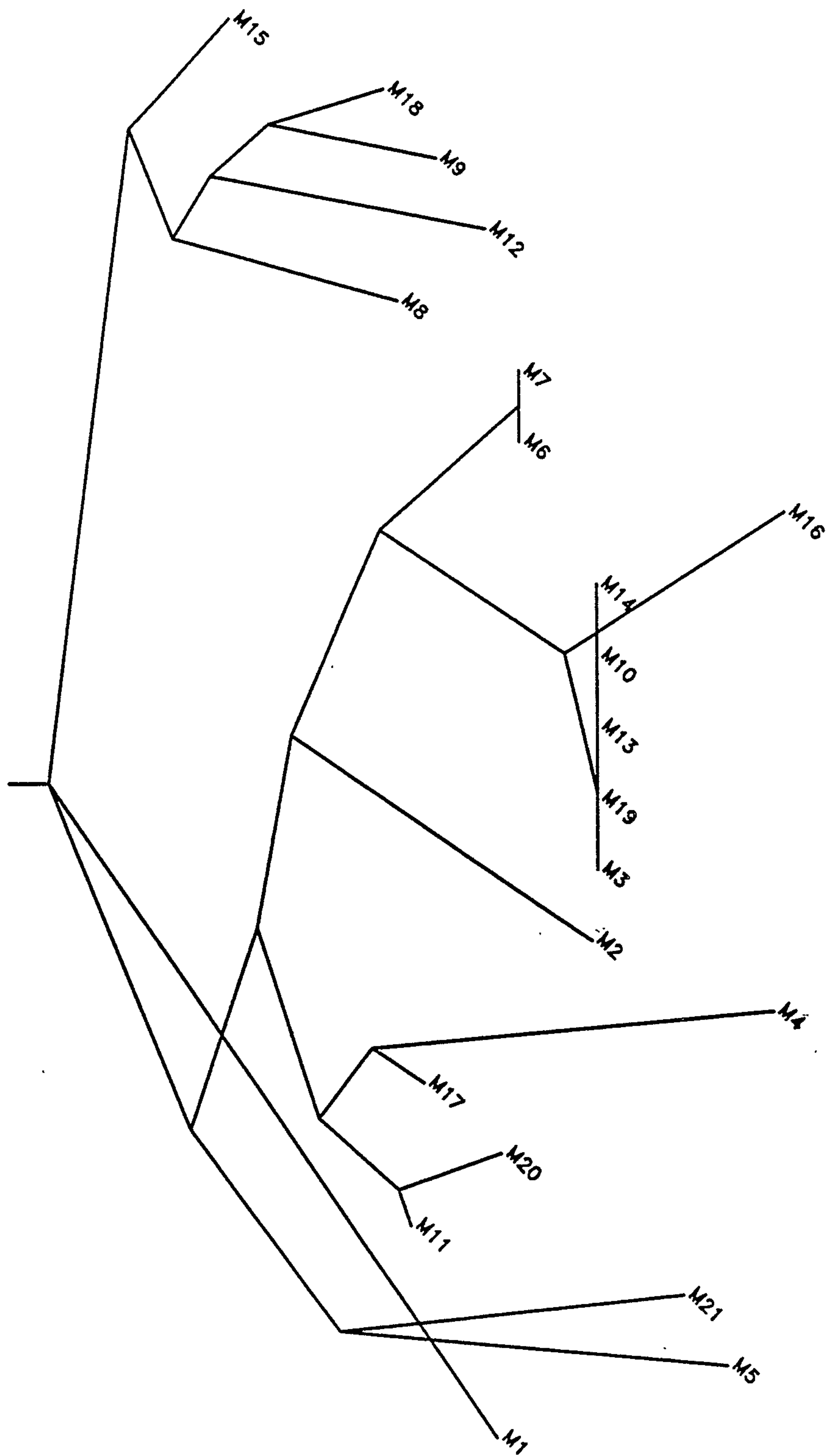


Fig. 6.8. Phylogenetic tree constructed from an alignment of the 21 'YG' repeats of GtfM.

6.3.3. Multiple Alignment and Phylogenetic Comparison with other Gtf Sequences

The amino acid sequence of GtfL and GtfM could be readily aligned with the nine previously sequenced GTFs. A multiple alignment of all eleven deduced amino acid sequences was constructed using default parameters of the 'ClustalV' program (Fig. 6.9). All the GTF proteins contain a typical streptococcal signal sequence, i.e. a basic N-terminal region followed by a hydrophobic region and a polar C-terminal region with a total length of 34-38 amino acids. Residues within the signal sequence were highly conserved allowing this region to readily align. The signal sequence is followed by a region of approximately 200 amino acids which is poorly aligned. A role for this region has not been determined. However N-terminal deletions of GtfL (this study) and GtfJ (Giffard and Jacques, unpublished observations) extending to different degrees into this region have no effect on enzyme activity. In the case of GtfL the 4470bp open reading frame is not entirely encoded on the 6.2kbp *Bam*HI/*Xba*I fragment. However, the phagemid, pGSG401, containing this fragment encodes for a protein with a 35-36 amino acid deletion of the predicted mature form of the protein that still possesses both sucrase and polymer forming activities (Section 5.3.4). In the case of the GtfJ, a far more extensive 122 amino acid deletion of the mature form of the protein does not appear to alter the properties of the enzyme (Giffard and Jacques, unpublished observations).

The region surrounding the putative active site was also highly conserved. The active site peptides of *S. sobrinus* GTF-S and GTF-I have been isolated by pepsin digestion of glucosyl-enzyme complexes (Mooser *et al.*, 1991). Site-directed mutagenesis has confirmed the importance of an aspartic acid residue, D⁴⁵¹ in forming a complex with glucose in the GtfB of *S. mutans* GS5 (Kato *et al.*, 1992). Comparison of the putative active sites of all the GTFs indicate that the amino acid sequences are very similar (Fig. 6.10) and that where differences occur they are generally conservative substitutions. The region containing the active site aspartic acid residue of the GtfL is therefore likely to be DGVRVDAVD, and that for GtfM DGVRIDAVD.

Hanada *et al.* (1993) have recently suggested that residues surrounding the active site aspartic acid may determine the nature of the linkages in the glucans

Fig. 6.9. Multiple alignment of the deduced amino acid sequence of the eleven available Gtfs of oral streptococci. The symbol '*' indicates positions of identity and '.' conserved amino acids.

GtfJ	MENKIHVYKLHKVKKQWVTIAVASVALATV LGG-----LSVTTSSVSADETQDKTVTQSNS
GtfK	MENKVRVYKLHKVKKQWVTIAVASVALATV LGG-----SVATSSSLASAEETNNSNGSPSTT
GtfL	MDKKVHYKMHKVKKQWVTIAVTGLSLGAVSAVSLGTNDGVVQADEHTDATVAIPDITVDT
GtfM	MENKVRFKLHKVKKNWVTIGVTTLSMVALAGGSLLAQGKVEADETSAPNGDGLQQLSE DG
GtfB	MDKKVRVYKLRKVKKRWVTVSVASAVMTL-----TTLSGGLVKADS NESKSQISNDS
GtfC	MEKKVRFKLRKVKKRWVTVSIA SAVVTL-----TSLSGSLVKADSTDDRQQAVTES
GtfD	METKRRYKMHKVKKHWVTVAVAS-GLITLGT TTL-----
GtfI (dn)	MEKNERFKMHKVKKRWVTISVASATMLA-----SALGASVASADTET----VSEDS
GtfS	MEKNLRYKLHKVKKQWVAIGVTTVTLSFLAGGQV-----VAADT
GtfI (sb)	MEKNVRFKMHKVKKRWVTVSVASATMLA-----SALGASVASADTDT----ASDDS
GtfT	MERKLHYKLHKVKKQWVTIAVASAGLASIVGA-----GSVGTQTVSADDLAQDQSSASEQK

*.***** **

GtfJ	GTTASLVTSPEATKEADKRTNTKEADVLT PAKETNAVETATTTNTQATAEAATTATTADV
GtfK	TVGEN--TNPVVEKEVGTTTEVANT---SNATTTERAEVTADKPAGTTVQPNSGTTSDRA
GtfL	GTVSNDTTAAQDPTTAVAATNDVATDQATPTATFDLTTD-----TTNTVAANAVDTVAT
GtfM	TASLVTTTTTVTEQASAQASVS AVATASVSHETSFOAATS AVSQEATAQAQTS PVASQEVA
GtfB	NTSVVTANEES-----NVITEATSK-----QEAASSQTNHTVTTSSSST
GtfC	QASLVTTSEAAKETLTATDTSTATSAT-----SQPTATVTDNVSTTNQSTN
GtfD	-----GSSVSAETEQQTSDKVVTKSEDDKAASESSQTDAP
GtfI (dn)	NQAVLTADQTTTNQDTEQTSVAATATS-----EQSASTDAATDQASATDQA
GtfS	NNNDGTSVQVNKMVPSDPKFDAQA QNGQLAQAMFKAANQA-DQTATSQVSPATDGRVDNQ
GtfI (sb)	NQTVVTGDQTTNNQATDQTSIAATATS-----EQSASTDAATDQ-----A
GtfT	AASDQ--KQEEVASDAANTASAK----VTSEKEVSQASDTTSESSQVQSQDQASAK--QA

GtfJ	AVAAVPNKEAVVTTDAPAVTTEKAEEQ PATVKAEVVNTEVKA-----
GtfK	AAVEVEAKPETTAKPEVATK PETATTSEVAANAGVAAPTTEK-----
GtfL	VGTDRAATTNDTTATNDTAVDTTNNNTTTD TTTVTDRAATTERR-----ATGARRG
GtfM	VSSQTQSSGQETQTTEQVSQ GQTSTQVAGQTS AQSTPSVTEQARPRVLTNAAPAIATRAA
GtfB	SVVNPKEVVSNPYTVGETAS--NGEKLQ NQTTTVDKTSEA-----
GtfC	TTANTANFVVKPTTTTSEQAKTDNSDKI ITTSKAVNRLTATGKFVP-----
GtfD	KTKQAQT--EQTQAQSQANVADTSTSITKETPSQ-----
GtfI (dn)	SAAEQTQGTTASTDTAAQTTTNANE-----AKWVP-----
GtfS	VTPAANQPAANVANQDVANPATDAGALNRQSAADTSTDG-----KAVPQTS DQ--
GtfI (sb)	SAAEQTQGTTASTDTAAQTTTNANE-----AKWVP-----
GtfT	SDLAPATKAAPQAESQAGTSTASSE AAPAASSQEVA----KS-----

GtfJ	-----PEAALKDSEV-----
GtfK	-----SKE-LSEAEI-----
GtfL	PTGGR----RATPVNGNTNNANNTVTVVNNDLPATNNVVTDG-----
GtfM	DSTIRINANRNTNITITASG TTPNVTIITGPNTPKPNVTVTSPNGTRPNVTIVTQPNQPN
GtfB	-----AANNISKQTTEADTDVIDDSNAANLQ-----ILEKLP-
GtfC	-----ANNNTAHPKTVTDKIVPIKPKIGKLKQPSSLSQDDIAALGNVK-
GtfD	-----NITTQANSDDKTVT----NTKSEE AQTSEERTKQSEEAQT TASSQAL
GtfI (dn)	-----TENE---NQVFTDEMLAEAKNVATAESNSIPSD---LAKMS-
GtfS	-----
GtfI (sb)	-----TENE---NQGFTDEMLAEAKNVATAESDSIPSD---LAKMS-
GtfT	-----TTGSVVSQNEE-----

GtfJ	-----EAALSLKNIK---NI-DGKY YVVEDGSHKENFAITVNGQLL
GtfK	-----KAAVSLDNIK---KEKDGKY YLLEDGSHKKNFAITVNGQVL
GtfL	-----PSHIKTINGKQY YVEDDGTIRKNYVLERIGGSQ
GtfM	KPVQPSQPSQPNKPVQPNQPSLDYKPVASNLKTIDGKQY YVE-NGVVKKNA AIELDGRLY
GtfB	-----NVKEIDGKY YYDNNGKVRTNFTLIADGKIL
GtfC	-----NIRKVNGKY YYYKEDGTLQKNYALNINGKTF
GtfD	TQAKAELTKQRQTAAQENKNPVDLAAIP-NVKQIDGKY YYIGSDGQPKKNFALT VNNKVL
GtfI (dn)	-----NVKQVDGKY YYYDQDGNVKKNF AVSVGEKIY
GtfS	-----PGHLETVDGKTY YVDANGQRLKNYSMVIDGKTY
GtfI (sb)	-----NVKQVDGKY YYYDQDGNVKKNF AVSVGDKIY
GtfT	-----AAALSLANIK---KI-DGKFY YVMADGSYKKNFAITVDGQML

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Gt f J	YFG-KDGALTSSSTYSFTPGTTNIVDG-----FSINNRAYDSSEASFELIDGYLTA
Gt f K	YFDEN-GALSSTSTYSFTQETTNLVTD-----FTKNNAAYDSTKASFELVDGYLTA
Gt f L	YFNAETGELSNQKEYRFDKNGGTGSSADSTNTNVTVNGDKNAFYGTDDKDIELVDGYFTA
Gt f M	YFD-ETGAMVDQSKPLYRADAI PNNSIYAVYNQA-----YDTSSKSFEHLDNFLTA
Gt f B	HFD-ETGAYTDT-----IDTVNKDIVTT-RSNLYKKYNQVYDRSAQSFEHVDHYLTA
Gt f C	FFD-ETGALSNT-----LPSKKGNITNNDNTNSFAQYNQVYSTDVANFEHVDHYLTA
Gt f D	YFDKNTGALTDTSQYQFKQGLTKLNNDYTPHNQI-----VNFENTSLETIDNYVTA
Gt f I (dn)	YFD-ETGAYKDTSK-----VEADKSGSDISKEETTFAANNRAYSTSAENFEAIDNYLTA
Gt f S	YFDGQTGE-----AQTDLPKTGQANQDNVPDSYQANNQAYSNEASSFETVDNYLTA
Gt f I (sb)	YFD-ETGAYKDTSK-----VDADKSSSAVSQNAITFAANNRAYSTSAKNFEAVDNYLTA
Gt f T	YFDAKTGALSSTSTYSFSQGLTPIVSD-----FSVNNKAFFDSSEKSFEHVDGYLTA
	* * * * *
Gt f J	DSWYRPASIIKDGVTWQASTAEDFRPLLMAWWPNVDTQVNYLNYMSKVFNLDK----YS
Gt f K	DSWYRPKEILEAGTTWKASTEKDFRPLLMWWPDKDTQVAYLNYMTKALSNGEETKDVFT
Gt f L	NTWYRPKEILKDGKEWTASTENDKRPLLTWWPDKAIQASYLNYMKEQ---GLGTNQTYT
Gt f M	DSWYRPKQILKDGKNWTASTEKDYRPLMTWWPDKVTQVNYLNYMSQQ---GFGN-KTYT
Gt f B	ESWYRPKYILKDGKTWTQSTEKDFRPLMTWWPDKQETQRYVNYMNAQ---LGINKTYD
Gt f C	ESWYRPKYILKDGKTWTQSTEKDFRPLMTWWPDKQETQRYVNYMNAQ---LGIHQTYN
Gt f D	DSWYRPKDILKNGKWTASSEDLRPLLMWWPDKQTQIAYLNYMNQ---GLGTGENYT
Gt f I (dn)	DSWYRPKSILKDGKWTESKDDFRPLLMWWPDTETKRNIVNYMNKV---VGIDKTYT
Gt f S	DSWYRPKILKNGQSWQASSEGLRPILMTWWPDAATKAAYANFWAKE---GL-ISGSYR
Gt f I (sb)	DSWYRPKSILKDGKWTESGKDDFRPLLMWWPDTETKRNIVNYMNKV---VGIDKTYT
Gt f T	ESWYRPAKILENGKTWVDSKETDLRPVLMWWPNKDTQVAYLNYMSKALGGKEE---FT
	..***** * . * * * * * * * * * * * * * .
Gt f J	STDKQETLKVAAKDIQIKIEQKIQAEKSTQWLRETISAFVKTPQWNKETENYSKGGGED
Gt f K	IENSQASLNAAAQILQRKIEVKIAANKSTDWLRQSIEAFVKDQDKWNINSE----SPGKE
Gt f L	SFSSQTQMDQAALEVQKRIEERIAREGNTDWLRRTTIKNFVKTPGWNSTSEN---LDNND
Gt f M	TDMMSYDLAAAAETVQRGIEERIGREGNTTWLRQLMSDFIKTPGWNSESEDNLLVG-KD
Gt f B	DTSNQLQLNIAAATIQAIEAKITTLKNTDWLRQTISAFVKTPQSAWNSDSEKPF---DD
Gt f C	TATSPLQLNLAAQTIQTKIEEKITAENNTWLRQTISAFVKTPQSAWNSDSEKPF---DD
Gt f D	ADSSQESLNLAQTVQVKIETKISQTTQWLRDIINSFVKTPQPNWNSQTESDTSAGEKD
Gt f I (dn)	AETSQADLTAAAEVLQARIEQKITTEQNTKWLREAI SAFVKTPQWNGESEKPY---DD
Gt f S	QNSA--NLDAATQNIQSAIEKKIASEGNTNWLRLDKMSQFVKSONQWSIASENETVYPNQD
Gt f I (sb)	AETSQADLTAAAEVLQARIEQKITSENNTKWLREAI SAFVKTPQWNGESEKPY---DD
Gt f T	TETSQTLTNTAAELIQAKIEARVSKEQGTKWLREAMAFAFVATQSRWNKDSEQYDKA---D
	. * . * * * * * * * * * * * * * .
Gt f J	HLQGGALLYVNDSDR-TPWANS DYRLNRTATNQTGTIDKSILDEQSDPNHMGGFDFLLAN
Gt f K	HFQKGALLFVNSDS-TKWANS DYRLNRTATSYI-KNHKIV-----NGSDGGYEFLLSN
Gt f L	HLQGGALLYVNDSDR-TSHANS DYRLNRTPTSQTGKHNP-----YTKDTSNGGFEFLLAN
Gt f M	HLQGGALTFLNNSA-TSHANS DFRLNRTPTNQTGT--RK-----YHIDRSNGGYELLAN
Gt f B	HLQNGAVLYDNEGKLT PYANS NYRI LNRTPTNQTGKKDPY--T--ADNTIGGYEFLLAN
Gt f C	HLQKGALLYSNNSKLT SQANS NYRI LNRTPTNQTGKKDPY--T--ADRTIGGYEFLLAN
Gt f D	HLQGGALLYSNSDK-TAYANS DYRLNRTPTSQTGK--PK-----YFEDNSSGGYDFLLAN
Gt f I (dn)	HLQNGALKFDNQSDLT PDTQSNYRLNRTPTNQTGSLDSRF--TYNANDPLGGYELLAN
Gt f S	HMQGGALLFSNSKD-TEHANS DWRLNRTPTFQTGKQK-----YFT-TNYAGYELLAN
Gt f I (sb)	HLQNGALLFDNQTDLT PDTQSNYRLNRTPTNQTGSLDSRF--TYNPNDPLGGYDFLLAN
Gt f T	HLQGGALLYTNNNL-TEWANS NWRLNRTPTRQDGKTH-----YSKADKYGGYEFLLAN
	* * * * * . * * * * * * * * * * * * * * * * * *
Gt f J	DVDLSNPVVQAEQLNQIHYLMNWGSIVMGDKD--ANFDGIRVDAVDNVDADMLQLYTNYF
Gt f K	DIDNSNPVVQAEMLNQLYYFMNWGQIVFGDKDKDAHFDGIRVDAVDNVSVDMLQLVSSYM
Gt f L	DIDNSNPVAVQAEQLNLWLHYIMNIGTIT--GGSEDENFDGVRVDAVDNVDADLLQIASDYF
Gt f M	DIDNSNPVAVQAEQLNLWLHYIMNIGSIL--GNDPSANFDGVRIDAVDNVDADLLQIASDYF
Gt f B	DVDNSNPVVQAEQLNLWLHFLMNFGNIY--ANDPDANFDSIRVDAVDNVDADLLQIAGDYL
Gt f C	DVDNSNPVVQAEQLNLWLHFLMNFGNIY--ANDPDANFDSIRVDAVDNVDADLLQIAGDYL
Gt f D	DIDNSNPVVQAEQLNLWLHYLMNYGSIV--ANDPEANFDGVRVDAVDNVDADLLQIASDYL
Gt f I (dn)	DVDNSNPVQAEQLNLWLHYLLNFGTIY--AKDADANFDSIRVDAVDNVDADLLQISSDYL
Gt f S	DVDNSNPVVQAEQLNLHLHYLMNWGDIV--MGDKDANFDGVRVDAVDNVDADLLQIQRDY
Gt f I (sb)	DVDNSNPVVQAEQLNLWLHYLLNFGSIY--ANDADANFDSIRVDAEDNVDADQLQISSDYL
Gt f T	DVDNSNPVVQAEMLNQIHYLMNWGEIVMGDKN--ANFDGIRVDAVDNVDADTLQLYTNYF
	* . *

Gt fJ	REYYGVNKSEANALAHISVLEAWSLNDNHYNDKTDGAALAMENKQRLALLFSLAKPIKE-
Gt fK	KAAYKVNESEARALANISILEAWSHNDPYVNEHNTAALSMDNGLRLSIVHGLTRPVTNK
Gt fL	KAKYGADQSQDQAIKHLISILEAWSHNDAYYNEDTKGAQLPMDDPMHLALVYSLLRPI-GN
Gt fM	KEKYRVADNEANAIAHLSILEAWSYNDHQYNKDTKGAQLSIDNPLRETLLTTFLLR----
Gt fB	KAAKG I HKNDKAANDHLSILEAWSNDNTPYLHDDGDNMINMDNKLRLSLLFSLAKPL-NQ
Gt fC	KAAKG I HKNDKAANDHLSILEAWSYNDTPYLHDDGDNMINMDNRLRLSLLYSLAKPL-NQ
Gt fD	KAHYGVDKSEKNAINHLSILEAWSNDNPQYNKDTKGAQLPIDNKLRLSLLYALTRPL-EK
Gt fI (dn)	KAAYGIDKNNKNANNHVSIVEAWSNDNTPYLHDDGDNLMNMDNKFRLSMLWSLAKPL-DK
Gt fS	KAKYGTQNEKNAIDHLSILEAWSGNDNDYVKDQNNFSLSIDNDQRSGLKAF-----
Gt fI (sb)	KAAYGIDKNNKNANNHVSIVEAWSNDNTPYLHDDGDNLMNMDNKFRLSMLWSLAKPT-DV
Gt fT	NSVYGVNKSEAQALAHISVLEAWSYNDNDYNQDTNGAALAMDNGLRFSLLYTLTRPINE-
	.. * ..*..***** ** *
Gt fJ	----RTPAVSPLYNNTF-NTTQRDEKTDWINKDGSKAYNEDGTVKQSTIGKYNEKYGDAS
Gt fK	GTGARNASMKDLINGGYFGLSNRAEVT-----SYDQLGF-----
Gt fL	-----RSGVEPLISNSLNRSE-----SGKNSKRM
Gt fM	----KSNYRGSLE RVITNSLNNRSS-----EQKHTPRD
Gt fB	-----RSGMNPLITNSLVNRTD-----DNAETAAV
Gt fC	-----RSGMNPLITNSLVNRTD-----DNAETAAV
Gt fD	DASNKNEIRSGLEPVITNSLNNRSA-----EGKNSERM
Gt fI (dn)	-----RSGLNPLIHNSLVDREV-----DDREVETV
Gt fS	--GYASAYRGNLSNLATAGLKNRSA-----NP-DSDPV
Gt fI (sb)	-----RSGLNPLIHNSLVDREV-----DDREVETV
Gt fT	----RTPGMSTLIKSEY-GLTDRTK-----NDKYGDTQ
	. *
Gt fJ	GNVVFIRAHDNNVQDIIAEI IKKEI-NPKSDGFT-ITDAEMKQAFEIYNKDMLSDDKKYT
Gt fK	ATYLFVRAHDSEVQTVIADIISKKI-DPTTDGFT-FTLDQLKQAFDIYNADMLKVDKEYT
Gt fL	ANYAFVRAHDSEVQSIIGQIIKNEI-NPQSTGNT-FTLDEMKKAFEIYNKDMRSANKQYT
Gt fM	ANYIFVRAHDSEVQAVLANIISKQI-NPKTDGFT-FTMDELKQAFEIYNADIADKKYT
Gt fB	PSYSFIRAHHDSEVQDLIADI I KAEI-NPNVVGYS-FTMEEIKKAFEIYNKDLLATEKKYT
Gt fC	PSYSFIRAHHDSEVQDLIRNIIRTEI-NPNVVGYS-FTTEEIKKAFEIYNKDLLATEKKYT
Gt fD	ANYIFIRAHHDSEVQTVIAKIIKQI-NPKTDGLT-FTLDELKQAFKIYNEDMRQAKKKYT
Gt fI (dn)	PSYSFARAHHDSEVQDLIRDI I KAEI-NPNAFGYS-FTQDEIDQAFKIYNEDLKKTDKKYT
Gt fS	PNYVFIRAHHDSEVQTRIAKIIREKLGKTNADGLTNLTLDLNLKAFDIYNQDMNATDKVYY
Gt fI (sb)	PSYSFARAHHDSEVQDIIRDI I KAEI-NPNSFGYS-FTQEEIDQAFKIYNEDLKKSDKKYT
Gt fT	PSYVFVRAHDSEVQTVIAQIIKEKI-DPTTDGFT-FTLDQLKQAFEIYNKDMNSVNXHYT
	* * ***** ** . * . * . * . * . * . *
Gt fJ	LNNIPAAAYAVMLQNMETITRVYYGDLYTDDGHYMETKSPYYDTIVNLMKSRIKYVSGGQ-
Gt fK	HSNIPAAAYALMLQTMGAATRVYYGDLYTDNGQYMAKKSPYFDQITTLKARP KYVAGGQT
Gt fL	QYNIPSAAYALMLTHKDTVPRVYYGDMYTDDGQYMAQKSPYYDAIETLLKGRIRYAAGGQD
Gt fM	QYNIPAAAYATMLTNKDSITRVYYGDLFTDDGQYMAEKSPYYNAIDALLRARIKYVAGGQD
Gt fB	HYNTALSYALLLTNKSSVPRVYYGDMFTDDGQYMAHKTINYEAIETLLKARIKYVSGGQA
Gt fC	HYNTALSYALLLTNKSSVPRVYYGDMFTDDGQYMAHKTINYEAIETLLKARIKYVSGGQA
Gt fD	QSNIPATAYALMLSNKDSITRLYYGDMYSDDGQYMATKSPYYDAIDTLLKARIKYAAGGQD
Gt fI (dn)	HYNVPLSYTLLLTNKGSIPRVYYGDMFTDDGQYMANKT VNYDAIESLLKARMKYVAGGQA
Gt fS	PNNLPMAYAWMLQNKDTVTRVYYGDMYTDDGQYMATKTPFYNAIETLLKGRIRKYVAGGQA
Gt fI (sb)	HYNVPLSYTLLLTNKGSIPRVYYGDMFTDDGQYMANKT VNYDAIESLLKARMKYVAGGQA
Gt fT	HYNIPAAAYAVMLSNMESVTRVYYGDLFTDDGQYMASKSPYYDAINTLLRARIKYAAGGQI
	* . . * . * . * . * . * . * . * . * . * . *
Gt fJ	-AQRSYWLPTDGKMDNSDV-ELYRTNEVYTSVRYGKDIMTANDTEGSKYSRTSGQVTLVA
Gt fK	----SY----IHNLAGDGVSSAKDNKEVLVSVRYGQDLMSKTDTEGGKYGRNSGMLTLIA
Gt fL	MKVN-----YIGYGNTN-GWDAAGVLTSVRYGTGANSASDT-GTAETRNQGMVAVIS
Gt fM	MKVT-----KLNG-----YE-IMSSVRYGKGAEANQL-GTAETRNQGMVLVLT
Gt fB	MRNQ-----QVGNSE-----IITSVRYGKGALKATDT-GDRTTRTSGVAVIEG
Gt fC	MRNQ-----QVGNSE-----IITSVRYGKGALKATDT-GDRTTRTSGVAVIEG
Gt fD	MKIT-----YVEGDKSHMDWDYTGVLT SVRYGTGANEATDQ-GSEATKTQGMVAVITS
Gt fI (dn)	MQNY-----QIGNGE-----ILTSVRYGKGALKQSDK-GDATTRTSGVGVMG
Gt fS	VSYK-----Q-----DWS-SGILTSVRYGKGANSASDA-GNTETRNSGMALLIN
Gt fI (sb)	MQNY-----QIGNGE-----ILTSVRYGKGALKQSDK-GDATTRTSGVGVMG
Gt fT	MEHNSYKPSVAMKAAHPDAGNVLGNSEVLVSVRFGQDVMSADDMTGKLAKTSGMFTLLIS
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Gt f J	NNPKLNLDQSAKLNVMGKIHANQKYRALIVGTADGIKNFTSDADAIAAGYVKETDSNGV
Gt f K	NNPDLKLADGETITVNMGAAHKNQAYRPLLLGTEKGIVSSLNDSDTKIVKY--TDAQGN
Gt f L	NQPALRLTSN--LTINMGAAHRNQAYRPLLLTTNDGVATYLNDS-AN-GIVKYTDGNGN
Gt f M	NRPDMKLGANDRLVVNMGAAHKNQAYRPLLLSKSTGLATYLNKSDVPA-GLVRYTDNQGN
Gt f B	NNPSLRLKASDRVVNMGAAHKNQAYRPLLLTTDNGIKAYHSDQEA--AGLVRYTNDRGE
Gt f C	NNPSLRLKASDRVVNMGAAHKNQAYRPLLLTTDNGIKAYHSDQEA--AGLVRYTNDRGE
Gt f D	NNPSLKLQNNDKVIIVNMGAAHKNQAYRPLLLTTKDGLTSTYTSDA- AK-SLYRKTNDKGE
Gt f I (dn)	NQPNFSLDGKV-VALNMGAAHANQEYRALMVSTKDG VATYATDADASKAGLVKRTDENG
Gt f S	NRPNFR--AYRNLTLMGAAHKSQAYRPLLLSTKDG IATYLNDS-DSRQYKYTDSQGN
Gt f I (sb)	NQPNFSLDGKV-VALNMGAAHANQEYRALMVSTKDG VATYATDADASKAGLVKRTDENG
Gt f T	NNPELELDVNEEIKVNVGKIHAGQAYRPLLLTTDKGLQKYLNDSDTKLT--KVADKDG
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Gt f J	LTFGANDIKGYETFDMSGFVAVWVPVGASDNQ--DIRVAPSTEAKKEGELTLKATEAYDS
Gt f K	LVFTADEIKGFKTVDMGYSVWVPVGATDDQ--NVLAKPSTKAYKEGDKVYSSAAL
Gt f L	LTFSANEIRGIRNPQVDGYLAVWVPVGASENQ--DVRVAPSKEKNSSGL-VYESNAALDS
Gt f M	LTFTADDIAGHSTVEVSGYLAVWVPVGASENQ--DARTKASSTKKGE-Q-VFESSAALDS
Gt f B	LIFTAADIKGYANPQVSGYLGWVPVGAALIKMFALRLAR--PHQOMA-SVHQNAALDS
Gt f C	LIFTAADIKGYANPQVSGYLGWVPVGAALIKMFALRLAR--PHQOMA-SVHQNAALDS
Gt f D	LVFDASDIQGYLNPQVSGYLGWVPVGAADNQ--DVRVAASSTAPSTDGK-SVHQNAALDS
Gt f I (dn)	LYFLNDDLKGVANPQVSGFLQVWVPVGAADDQ--DIRVAASDTASTDGK-SLHQDAAMDS
Gt f S	LSFSASELQSVANAQVSGMIQVWVPVGAADNQ--DVRTSPSTQATKDG- IYHQSDALDS
Gt f I (sb)	LYFLNDDLKGVANPQVSGFLQVWVPVGAADDQ--DIRVAASDTASTDGK-SLHQDAAMDS
Gt f T	ITFKGSEIKGYKQVEVNGYLSVWVPVGAADQ--DIRVAPSTAACKGEKDKTYTASQAL
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Gt f J	QLIYEGFSNFQTI PDGSDPSVYTNRKIAENVDLFKSWGVTSFEMAPQFVSADDGTFLDSV
Gt f K	QVIYEGFSNFQDFVK--EDSQYTNKLIANADLFKSWGITSFEIAPQYVSSKDGTFLLDSI
Gt f L	QVIYEGFSNFQDFVQ--NPSQYTNKKIAENANLFKSWGITSFEFAPQYVSSDDGSFLDSV
Gt f M	QVIYEGFSNFQDFVK--TPSQYTNRVIAQNAKLFEWGVTSFEFAPQYVSSQDGTFLDSI
Gt f B	RVMFEGFSNFQAFAT--KKEEYTNVVIKAVNDKFAEWGVTD FEMAPQYVSSDTGGSFLDSV
Gt f C	RVMFEGFSNFQAFAT--KKEEYTNVVIKAVNDKFAEWGVTD FEMAPQYVSSDTGGSFLDSV
Gt f D	QLIYEGFSNFQDFVT--KDSYTNKKIAQNVQLFKSWGVTSFEMAPQYVSSDGTGFLDSI
Gt f I (dn)	RVMFEGFSNFQSFAT--KEEYTNVVIANNVDKFSWGVTD FEMAPQYVSSDTGQFLDSV
Gt f S	QVIYEGFSNFQAFQ--SPDQYTNVIAKNGDLFKSWGVTD FEMAPQYVSSDGTGFLDSV
Gt f I (sb)	RVMFEGFSNFQSFAT--KEEYTNVVIANNVDKFSWGVTD FEMAPQYVSSDTGQFQDSV
Gt f T	QLIYEGFSNFQDFVQ--KDSQYTNKKIAENTDLFKAWGVTSFEMAPQYVSATDGTFLDSI
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Gt f J	IQNGYAFADRYDLAMSKNNKYGSKEDLRDALKALHKAGIQAIADWVPDQIYQLPGKEVVT
Gt f K	IENGYAFTDRYDFAMSKNNKYGSKEDLRDALKALHKQGIQVIADWVPDQLYTLPGKEVVT
Gt f L	IQNGYAFADRYDIGMSKDNKYGSLADLKAALKSLHAVGISAIADWVPDQIYNLPGDEVVT
Gt f M	IENGYAFEDRYDIAMSKNNKYGSLKDLMDALRALHAEGISAIADWVPDQIYNLPGKEVVT
Gt f B	IQNGYAFADRYDLGISKPNKYGTADDLVKAIKALHSGIKVMADWVPDQMYALPEKEVVT
Gt f C	IQNGYAFADRYDLGISKPNKYGTADDLVKAIKALHSGIKVMADWVPDQMYALPEKEVVT
Gt f D	IQNGYAFEDRYDLAMSKNNKYGSQDQMINAVKALHSGIQVIADWVPDQIYNLPGKEVVT
Gt f I (dn)	IQNGYAFADRYDLGMSKANKYGTADQLVKAIKALHAKGLKVMADWVPDQMYTFPKQEVVT
Gt f S	ILNGYAFSDRYDLAMSKNNKYGSKQDLANAIKGLQSAGIKVLSLVPNQLYNLPGKEVVT
Gt f I (sb)	IQNGYAFADRYDLGMSKANKYGTADQLVKAIKALHAKGLKVMADWVPDQMYTFPKQEVVT
Gt f T	IENGYAFTDRYDLAMSKNNKYGSKEDLANALKALHAAGIQAIADWVPDQIYQLPGKEVVT
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Gt f J	ATRTDGAGRKIADAIIDHSLYVANSKSSGKDYQAKYGGEF LAELKAKYPEMFKVNMISTG
Gt f K	ATRTDTHGKVLDDTSLVNKLYVTNTKSSGNDFQQAQYGGAF LDKLQKLYPEIFKEVMEASG
Gt f L	ATRVNNYGETKDGAIIIDHSLYAAKTRTFGNDFQGKYGGAF LDELKRLYPQIFDRVQISTG
Gt f M	ASRTNSYGTTPRPAEIYNSLYAAKTRTFGNDFQGKYGGAF LDELKAKYPAIFERVQISNG
Gt f B	ATRVDKYGTPVAGSQIKNTLYVVDGKSSGKDQQAQYGGAF LEELQAKYPELFARKQISTG
Gt f C	ATRVDKYGTPVAGSQIKNTLYVVDGKSSGKDQQAQYGGAF LEELQAKYPELFARKQISTG
Gt f D	ATRVNDYGEYRKDSEIKNTLYAANTKSNGKDYQAKYGGAF LSELAAKYPSIFNRTQISNG
Gt f I (dn)	VTRTDKFGKPIAGSQINHSLYVTDTKSSGDDYQAKYGGAF LDELKEKYPELFTKKQISTG
Gt f S	ATRVNQYGOAKSGATINKTPYVANTRSYG-DYQEQYGGKFL DDLQKLYPRLFSTKQISTG
Gt f I (sb)	VTRTDKFGKPIAGSQINHSLYVTDTKSSGDDYQAKYGGAF LDELKEKYPELFTKKQISTG
Gt f T	ASRVDNYGRVKVDQPLVEKLYLANTKSSGKDFQAKYGGEF LAELQKKYPEMFTTKMISTG
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Gt f J	KPIDDSVKLKQWKA EYFNGT NVL ERGVGYVLSDEATGKYFTVTKEG--NFIP LQLTGKEK
Gt f K	KTIDPSVKIKQWEAKYFNGTNIQKRGSDYVLS DGKL--YFTVNDKG--TFLPAALTGDTK
Gt f L	KRMTTDEKITQWSAKYMNGTNI LDRGSEYVLKNGLNG--YYGTNGGKV--SLPKVVGSNQS
Gt f M	RKLTTNEKITQWSAKYFNGSNIQGTGARYVLQDNATNQYFSVKAGQT--FLPKQMT EITG
Gt f B	VPMDPSVKIKQWSAKYFNGTNI LGRGAGYVLKDQATNTYFNISDNKEINFLPKTLLNQD-
Gt f C	VPMDPSVKIKQWSAKYFNGTNI LGRGAGYVLKDQATNTYFSLVSDNT--FLPKSLVNP NH
Gt f D	KKIDPSEKITAWKAKYFNGTNI LGRGVGYVLKDNASDKYFELKGNQT--YLPKQMTNKEA
Gt f I (dn)	QAIDPSVKIKQWSAKYFNGSNI LGRGADYVLS DQASNKYLVNSDDKL--FLPKTLLGQ--
Gt f S	KPIDPSVKITNWSAKYFNGSNI LGRGAKYVLSE--GNKYLVNLADGKL--FLPTVL-----
Gt f I (sb)	QAIDPSVKIKQWSAKYFNGSNI LGRGADYVLS DQVSNKYFNVASDTL--FLPSSLLGK--
Gt f T	KTIDPSVKLKQWSAKYFNGT NVL DRGTDYILSDEGTGKYFTVNEKG--DFLPASLTGKKD
	. * . * * * * . * * . * . * .
Gt f J	VIT-----GFSSDGKGITYF-GTSGTQAKSAFVTFNGNT-YYFDARGHMVTNSEYS
Gt f K	AKT-----GFAYDGTGVTTY-TTSGTQAKSQFVTYNGKQ-YYFNDKG YLVTGEQTI
Gt f L	TNGDNQNGDGSGKFEKRLFSVR YRYNNGQY-AKNAFIKDNDGNVYFYFDSGRMAVGEKTI
Gt f M	S-GFRRVGDD-----VQY-LSIGGYLAKNTFIQVGANQWYFYFDKNGNMVTGEQVI
Gt f B	----SQVGF--SYDGKGY--VYYS-TSGYQAKNTFISEG-DKWYFYFDNNGYMTGAQSI
Gt f C	GTSSSVTGL--VFDGKGY--VYYS-TSGNQAKNAFISLG-NNWYFYFDNNGYMTGAQSI
Gt f D	STGFVNDGNG-----MTF-YSTSGYQAKNSFVQDAKGNWYFYFDNNGHMYGLQQL
Gt f I (dn)	---VVESGI---RFDGTGY--VYNSSTTGEKVTD SFITEA-GNLYYFGQDGYMTGAQNI
Gt f S	NNTYGPQVSANGFISKNGGIHY-LDKNGQEVKNRF-KEISGSWYFYFSDGKMATGKTKI
Gt f I (sb)	---VVESGI---RYDGKGY--IYNSSATGDQVKASFITEA-GNLYYFGKDG YMTGAQTI
Gt f T	AKT-----GFYNDGKGIVYY-TTAGNKAKSAFVTEAGNT-YYFDYTGHMVTGPNVI
	. * * * * *
Gt f J	PNGKDVYRFLPNGIMLSNAFYIDANGNTYLYNSKGQMYKGGYTKFDVSETDKDGKESKVV
Gt f K	DGSN--YFFLPNGVMFTDGV RKNAGQSLVYGKSGKLTQTGWKE-VTVKDDSGKEEF-
Gt f L	DGKQ--YFFLANGVQLRDGYRQNR RGQVFYYDQNGV-----
Gt f M	DGKK--YFFLDNGLQLRHVLRQGS DGHVYYDPKGVQAFN--GFYDFAGPRQD-----
Gt f B	NGVN--YYFLSNGLQLRDAILKNEDGT YAYYGNDRRYEN--GYQFMSGV-----
Gt f C	NGAN--YYFLSNGIQLRNAIYDNGNKVLSYYGNDRRYEN--GYLFGQ-----
Gt f D	NGEV--QYFLSNGVQLRESFLENADGSKNYFGHLGNRYSN--GYYSFDNDSK-----
Gt f I (dn)	KGSN--YYFLANGAALRNTVYTDAQ GQNHYGNDGKRYEN--GYQQFGNDS-----
Gt f S	GNDT--YLEMPNGKQLKEGVWYDGK-KAYYYDDNGRTWTN-KGFVEFRVDGQD-----
Gt f I (sb)	NGAN--YFFLENGTALRNTIYTDAQ GNSHYANDGKRYENENGYQQFGND-----
Gt f T	--NTKFYYFLPNGVMLKDAVMQDS KGRSVYYGKTGVMYKATENSEWFAMTDDKGQ---V
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Gt f J	KFRYFTNEGVMAGVTVIDGFTQYFGED-GFQAKDKLVTFK-GKTTYFDAHTGNGIKDTW
Gt f K	-YQYFFKGGIMATGLTEVEGKEKYFYDN-GYQAKGVFVPTKDGHLMFFCGDSGERKYSGF
Gt f L	----LNANGKQ-----DPKPDNNNNASGRNQFVQIGN-----
Gt f M	-VRYFDGNGQMYRGLHDMYGTTFYFDEK TGIQAKDKFIRFADGRTRYFIPDTGNLAVNRF
Gt f B	-WRHFN-NGEMSVGLTVIDGQVQYFD-EMGYQAKGKFVTTADGKIRYFDKQSGNMYRNRF
Gt f C	-WRYFQ-NGIMAVGLTRVHGAVQYFD-ASGFQAKGQFITTADGKLRYFDRDSGN-----
Gt f D	-WRYFDASGVMAVGLKTINGNTQYFDQD-GYQVKGAWITGSDGKKRYFDDGSGNMAVNRF
Gt f I (dn)	-WRYFK-NGVMALGLTTVDGHVQYFD-KDGVQAKDKIIVTRDGKVR YFDQHNGNAVTNTF
Gt f S	KWRYFNGDGTIAIGLVSLD-----
Gt f I (sb)	-WRYFK-DGNMAVGLTTVDGNVQYFD-KDGVQAKDKIIVTRDGKVR YFDQHNGNAVTNTF
Gt f T	RFRHFDRYGFM SIGLV TINQNVQYYDEN-GFQVKGEFVTDKDGQTRYFDQGS GNLVKGQF
	. *
Gt f J	RNINGK--WYYFDANGVAATG-----
Gt f K	FEQDGN--WYYANDKGYVATGFTKV GKQNL YFNEKGVQVKNRFFQVG DAT-----
Gt f L	-----NVWAYYDGN GKRV TGHQNINGQELFFDNNGVQVKGR TVNENG AIRYY-DANSGE
Gt f M	AQNPENKAWYYLDSNGYAVTGLQTINGKQY YFDNEGRQVKGHFVTINNQ-RYFLDGD SGE
Gt f B	IENEEGK-WLYLGEDGA AVTGSQTINGQHLYFRANGVQVKGEFVTDH HGRI SYDGN SGD
Gt f C	-----
Gt f D	ANDKNGD-WYYLNSDGIALVGVQTINGKTY YFGQDGKQIKGKIITDNGKLYFL-ANSGE
Gt f I (dn)	VADKTGH-WYYLGKDGVAVTGAQTVGKQHLYFEANGQQVKGDFVTA KDGLYFYD VDSGD
Gt f S	-----NRTLYFDAYGYQVKGQTV TIN-GKSYTFDADQGD
Gt f I (sb)	IADKTGH-WYYLGKDGVAVTGAQTVGKQKLYFEANGQQVKGDFVTSDEGKLYFYD VDSGD
Gt f T	LNKDGN--WYYLDDNGLVAKG-----

Gt fJ	-----AQVINGQKLYFNED-GSQVKGGVVKNADGTYSKY
Gt fK	-----YYANNEGDVLRGAQTINGDELYFDE-SGKQVKGEFVNNPDGTTSY
Gt fL	MARNRFAEIEPGVWAYFNNDGTAVKGSQNINGQDLYFDQN-GRQVKGALANV-DGNLRY
Gt fM	IAPSRFVTEN-NKYYYVDGNGKLVKGAQVINGNHYYFNND-YSQVKGAWANG-----RYY
Gt fB	QIRNRFVRNAQGQWFFYFDNNGYAVTGARTINGQLLYFRAN-GVQVKGEFVTDYGRISYY
Gt fC	QISNRFVRNSKGEWFLFDHNGVAVTGTVTENGQRLYFKPN-GVQAKGEFIRDANGYLRY
Gt fD	LARNIFATDSQNNWYYFGSDGVAVTGSQTIAG-----
Gt fI (dn)	MWTNTFIEDKAGNWFYLGKDGA AVTGAQTIKGQKLYFKAN-GQQVKGDIVKDADGKIRYY
Gt fS	LVQ-----
Gt fI (sb)	MWTDTFIEDKAGNWFYLGKDGA AVTGAQTIRGQKLYFKAN-GQQVKGDIVKGTGDKIRYY
Gt fT	-----AQTVKGQKLYFDTKTG VQVKGDFVADKDGNLTFY

Gt fJ	KEGFG-ELVTN-----E-----FFT-----
Gt fK	DAITGVKLVDTSL-VVDGQTFNVDAGVVTKAHTPG-----FYT-----
Gt fL	DVNSGELYRNR FHE-----IDGS
Gt fM	DGDSGQAVSNQFIQ-----IAAN
Gt fB	DGNSGDQIRNRFVRNAQGQ-----
Gt fC	DPNSGNEVRNRFVRNSKGE-----
Gt fD	-----
Gt fI (dn)	DAQTGEQVFNKS V-SVNGKTY YFGSDGTAQTQANPKGQTFKDGSGVLRFY NLEGQYVSGS
Gt fS	-----TDNANPAPQGQA-----
Gt fI (sb)	DAKSGEQVFNKT VKAADGKTYVIGNDGVAVDPSVVKGQTFKDASGALRFYNLKGQLVTGS
Gt fT	SGDTG-ELVQS-----D-----FFS-----

Gt fJ	----TDGNVWYYAGANGKTVTGAQVINGQHLYFNAD-GSQVKGGVVKNADGT-YSKYNAS
Gt fK	----TGDNWVYADSYGRNVTGAQVINGQHLYFDAN-GRQVKGGFVTNTDGSR-SFYHWN
Gt fL	-WY-----YF-DGNGNAVKGMVNINGQNLLFDNN-GKQIKGHLVR-VNG-VVRYFDPN
Gt fM	QWA-----YL-NQDGHKVTGLQNINNKVYYFGSN-GAQVKGKLLT-VQG-KKCYFDAH
Gt fB	-WF-----YF-DNNGYAVTGARTINGQHLYFRAN-GVQVKGEFVTDRHG-RISYYDGN
Gt fC	-WF-----LF-DHNGIAVTGARVVNGH-----A-SILSLMV F-----
Gt fD	-----KKLYFASD-GKQVKGSFVT-YNG-KVHYHAD
Gt fI (dn)	GWYETAHEWVY-VKSGKVL TGAQTIGNQRVYFKDN-GHQVKGQLVTGNDG-KLRY YDAN
Gt fS	GWKLLGDNQWGY-RKD GQLLTGEQTIDGQKVFFQDN-GVQVKGGTAT-----
Gt fI (sb)	GWYETANHDWVY-IQSGKALTGEQTINGQHLYFKKD-GHQVKGQLVTGTDG-KVRY YDAN
Gt fT	----TGNNTWFYADENGHVAKAKTIRGQKLYFDTKTGQQA KGRFIRDDKGVRY--YDAD

Gt fJ	TGERLTNEFFT-----
Gt fK	TGDKLVSTFFATGHDRWYYADDRGNVVTGAQVINGQKLFFD TDGKQVKGAFATNANGSRS
Gt fL	SGEMAVNR-----
Gt fM	TGEQVVNR-----
Gt fB	SGDQIRNRFV-----
Gt fC	---RLRESSL-----
Gt fD	SGELQVNR-----
Gt fI (dn)	SGDQAFNKS VTVNGKTY YFGSDGTA-----QTQANPKGQTF-KDGSGVLR
Gt fS	-----DASGVLR
Gt fI (sb)	SGDQAFNKS VTVNGKTY YFGNDGTA-----QTAGNPKGQTF-KDGSDI-R
Gt fT	TGALVTNAFLET-----

Gt fJ	-----GDNNWYYIGANGKSVTGEVKIGDDTYFFAKD-GKQVKGQTVSA
Gt fK	YYHWNTGNKLVSTFFTSGDNNWYYADAKGEVVVGEQTINGQHLYFD-QTGKQVKGATATN
Gt fL	-----WVEVSPGWVYFDGEG-----
Gt fM	-----FVEAARGCWYYFNSAGQAVTGQVINGKQLYFD-GSGRQVKGRYV--
Gt fB	-----RNAQGQWFFYFDNNGYAVTGARTINGQHLYFR-ANGVQVKGEFVTD
Gt fC	-----QSVK-----VVSNTMILIP EMKF-----
Gt fD	-----FEADKDNWYYLDSNGEALTGSQRINDQRVFFT-REGKQVKGDVA--
Gt fI (dn)	FYNLEGQYVSGSGWYKNAQGQWLYV-KDGKVL TGLQTVGNQKVYFD-KNGIQAKGKAVRT
Gt fS	FYDRDQGHQVGKGWYSTSDDNWVYVNESGQVLTGLQTIDGQTVYFD-DKGIQAKGKAVWD
Gt fI (sb)	FYSMEGQLVTGSGWYSNAQGQWLYV-KNGKVL TGLQTVGSQRVYFD-ENGIQAKGKAVRT
Gt fT	-----KAGSNQWYYMGADGYAVKGNQTIKNQHMYFDAETGQQA KGIIVTD

GtfJ	GNGRISYYYGD-SGKRAVSTWIEIQPGVYVYFDKNGLA-YPPRVLN
GtfK	PDGSISY-YDVHTGEKAINRWVKIPSGQWVYFNAQG-KGYVSN---
GtfL	-----RGQI--
GtfM	-----YV-GGK-----RLFCDAKTGE--LRQRR--
GtfB	RYGRISYY-DANSGERVRIN-----
GtfC	-----VIVM-----
GtfD	-----Y--DER-----RLLVY-----R--
GtfI (dn)	SDGKVRYF-DENSGSMITNQWKVY-GQYYYFGSDGAAVYRGWN--
GtfS	ENGNLRYF-DADSGNMLRDRWKNVD-GNWYYFNRNG---LATRW--
GtfI (sb)	SDGKIRYF-DENSGSMITNQWKEVN-GRYYYFGNDGARIYRGWN--
GtfT	ANGR-KYFYDTFTGSRVVNQFVLVNGNWy-FFGYDGAAGYRYSRLC

<i>Streptococcus mutans</i> GtfB	446	D S I R V D A V D	454
<i>Streptococcus mutans</i> GtfC	482	D S I R V D A V D	491
<i>Streptococcus mutans</i> GtfD	467	D G V R V D A V D	475
<i>Streptococcus downei</i> GtfI	543	D S I R V D A V D	461
<i>Streptococcus downei</i> GtfS	443	D G V R V D A V D	451
<i>Streptococcus sobrinus</i> GtfI	442	D S I R V D A E D	450
<i>Streptococcus sobrinus</i> GtfT	446	D G I R V D A V D	454
<i>Streptococcus salivarius</i> GtfJ	466	D G I R V D A V D	474
<i>Streptococcus salivarius</i> GtfK	456	D G I R V D A V D	464
<i>Streptococcus salivarius</i> GtfL	501	D G V R V D A V D	509
<i>Streptococcus salivarius</i> GtfM	553	D G V R I D A V D	561

Fig. 6.10. Putative active-site peptide sequences of the Gtfs of oral streptococci. The numbers at each end of the peptide sequence indicated that first and last amino acid. The Asp site of the GtfB of *S. mutans* GS5 marked in bold has been determined to be essential for activity by site-directed mutagenesis.

produced by the different enzymes. This would seem unlikely, however, as GtfJ and GtfK have the same active site sequence, but produce glucans with different structures (Pitty *et al.*, 1989; Section 7). Regions preceding the active site are also thought to be important as antibodies raised against peptides derived from this region inhibit GTF activity, although their function has not been determined (Chia *et al.*, 1993b; Dertzbaugh and Macrina, 1990; see also Section 1.6).

The homologous region in the 'catalytic domain' was selected for further analysis. This region corresponds to residues 211-1098 of GtfL and residues 275-1145 of GtfM. The multiple alignment parameters that resulted in minimum gaps in a previous study (Giffard *et al.*, 1993) were found to hold in this extended analysis (6.2.2 above; Fig. 6.13), and were used to construct a phylogenetic tree (Fig. 6.14). Excluding the groupings of GtfB/GtfC and the GtfIs of *S. sobrinus* and *S. downei* (which are identical over much of their length), there was little clustering of enzymes with similar activities. This is in keeping with the results of Giffard *et al.* (1993) who found no clustering of enzymes producing soluble or insoluble glucans.

The phylogenetic tree (Fig 6.12) indicates that the two primer-independent GTFs of *S. salivarius* (GtfL and GtfM) group with the primer-independent GTF-S of *S. mutans* (GtfD), although they are not closely related. The primer-independent GTF-S proteins of *S. sobrinus* (GtfT) and *S. downei* (GtfS) were not positioned in this group. Interestingly, the GtfT of *S. sobrinus* was more closely related to the primer-dependent GTFs of *S. salivarius* (GtfJ and GtfK) than to its own GTF-I (GtfI), while the GtfS was to be found on a branch of its own. The primer-dependent GTFs of *S. salivarius*, GtfJ and GtfK, did not group with those of *S. mutans*, *S. downei* or *S. sobrinus*.

The C-terminal glucan-binding domain of the 11 GTFs also did not align. This was predictable considering previous observations (Giffard and Jacques, 1993, 1994), as this region of the proteins contains a large number of repeating units that have undergone a number of independent duplications and rearrangements (6.1; 6.3.1.3; 6.3.2.3 above).

Multiple alignments of the five primer-independent and six primer-dependent Gtfs were also constructed (data not shown). As the GtfB and GtfC

of *S. mutans* GS5 are strongly primer-activated when introduced into *S. milleri* (Fukushima *et al.*, 1992) they were grouped with the primer-dependent enzymes, although they appear primer-independent in *E. coli* (Aoki *et al.*, 1986; Hanada and Kuramitsu, 1988). Visual examination of these alignments indicated that there were no conserved features distinct to either group could account for primer-dependence.

Fig. 6. 11. Multiple alignment of the central portion of the deduced amino acid sequences of the 'catalytic' domains of the eleven available Gtfs. The constraints used in constructing the alignment are described in the text. The symbols '*' are positions of identity and '.' conserved amino acids. The amino acid residues aligned are: GtfJ, 180-1108; GtfK, 176-1078; GtfL, 211-2098; GtfM, 275-1145; GtfB, 166-1037; GtfC, 191-1075; GtfD, 166-1069; GtfI (dn), 168-1046; GtfS (dn), 156-1026; GtfI (sb), 162-1035; GtfT (sb), 168-1060.

Gt f J	DGKYYYVNEDGSHKENFAITVNGQLLYFG-KDGALTSSSTYSFTP GTTNIVDG-----
Gt f K	DGKYYYLLEDGSHKKNF AITVNGQVLYFDEN-GALSSTSTYSFTQETT NLVTD-----
Gt f L	NGKQYYVEDDGTIRKNYVLERIGGSQYFNAETGELSNQKEYRFDKNGGTGSSADSTNTNV
Gt f M	DGKQYYVE-NGVVKKNA AIELDGRLYYFD-ETGAMVDQSKPLYRADAI PNNSIYAVYNQA
Gt f B	DGKYYYDNNNGKVRTNFTLIADGKILHFD-ETGAYTDTS-----IDTVNKDIVTT-RS
Gt f C	NGKYYYYKEDGTLQKNYALNINGKTFFFD-ETGALSNT-----LPSKKGNITNNDNT
Gt f D	DGKYYYIGSDGQPKKNFALT VNNKVLYFDKNTGALTDTSQYQFKQGLTKLNNDYTPHNQI
Gt f I - dn	DGKYYYDQDGNVKKNF AFSVGEKIYYFD-ETGAYKDTSK-----VEADKSGSDISKEE
Gt f S	DGKTYVDANGQRLKNYSMVIDGKTYFDGQTGE-----AQTDLPKTGQANQDNVP
Gt f I - sb	DGKYYYDQDGNVKKNF AFSVGDKIYYFD-ETGAYKDTSK-----VDADKSSSAVSQNA
Gt f T	DGKFYYVMADGSYKKNF AITVDGQMLYFDAKTGALSSTSTYSFSQGLTPIVSD-----
	. ** * . * * . * *
Gt f J	--FSINN RAYDSSEASFELIDGYLTADSWYRPASIIKDGVTWQASTAEDFRPLLMAWWPN
Gt f K	--FTKNNAAYDSTKASFELVDGYLTADSWYRPKEILEAGTTWKASTEKDFRPLLMSWWPD
Gt f L	TVNGDKNAFYGTDDKDIELVDGYFTANTWYRPKEILKDGKEWTASTENDKRPLLTVWWPS
Gt f M	-----YDTSSKSFEHLDNFLTADSWYRPKQILKDGKNWTASTEKD YRPLLMTWWPD
Gt f B	NLYKKYNQVYDRSAQSFEHVDHYLTAESWYRPKYILKDGKTWTQSTEKDFRPLLMTWWPD
Gt f C	NSFAQYNQVYSTDVANFEHVDHYLTAESWYRPKYILKDGKTWTQSTEKDFRPLLMTWWPD
Gt f D	-----VNFENTSLETIDNYVTADSWYRPKDILKNGKTWTASSEDLRPLLMSWWPD
Gt f I - dn	TTFAANNRAYSTSAENFEAIDNYLTADSWYRPKSILKDGKTWTESKDDFRPLLMAWWPD
Gt f S	DSYQANNQAYSNEASSFETVDNYLTADSWYRPKRILKNGQSWQASSEGLRPILMTWWPD
Gt f I - sb	TIFAANNRAYSTSAKNFEAVDNYLTADSWYRPKSILKDGKTWTESGKDDFRPLLMAWWPD
Gt f T	--FSVNNKAFDSSEKSFELVDGYLTAESWYRPAKILENGKTWVDSKETDLRPVLMSWWPN
	. * . * . * . * . * . * . * . * . * . * . * . * . * . * . * . *
Gt f J	VDTQVNYLNYMSK-VFNLDKYSSTDKQETLKVAAKDIQIKIEQKIQAEKSTQWLRETIS
Gt f K	KDTQVAYLNYMTK-ALSNGEETKDVFTIENSQASLNAAAQILQRKIEVKIAANKSTDWLR
Gt f L	KAIQASYLNYMKEQGLGTNQTYSFSSQTQMDQAALVQKRIEERIAREGNTDWLRTTIK
Gt f M	KVTQVNYLNYMSQGGFGN-KTYTTDMMSYDLAAAETVQRGIEERIGREGNTTWLRQLMS
Gt f B	QETQRQYVNYMNAQ-LGINKTYDDTSNQLQLNIAAATIQA KIEAKITTLKNTDWLRQTIS
Gt f C	QETQRQYVNYMNAQ-LGIHQTYNTATSPLQLNLAAQTIQTKIEEKITAEKNTNWLRTIS
Gt f D	KQTQIAYLNYMNQGLGTGENYTADSSQESLNLAQAQTVQVKIETKISQTTQWLRLDIIN
Gt f I - dn	TETKRNYVNYMNKV-VGIDKTYTAETSQADLTAAAEVLQARIEQKITTEQNTKWLREAIS
Gt f S	AATKAAYANFWAKEGL-ISGSYRQNSA--NLDAATQNIQSAIEKKIASEGNTNWLRLDKMS
Gt f I - sb	TETKRNYVNYMNKV-VGIDKTYTAETSQADLTAAAEVLQARIEQKITSENNTKWLREAIS
Gt f T	KDTQVAYLNYMSK-ALGGKEEFTTETSQTLTLNTAAELIQAKIEARVSKEQGTKWLREAMA
	. * *
Gt f J	AFVKTQPQWNKETENYSKGGGEDHLQGGALLYVND SRTPWANS DYRRLNRTATNQTGTID
Gt f K	QSIEAFVKDQDKWNINSESPGKEHFQKGALLFVNSDSTKWANS DYRKLNTATS-----
Gt f L	NFVKTQPGWNSTSEN--LDNNDHLQGGALLYNND SRTSHANS DYRLLNRTPTSQTGKH
Gt f M	DFIKTQPGWNSESEDNLLVG-KDHLQGGALTFLNNSATSHANSDFRLMNRTPTNQTGT--
Gt f B	AFVKTQSAWNSDSEKPF--DDHLQNGAVLYDNEGKLTPYANSNYRI LNRTPTNQTGKKD
Gt f C	AFVKTQSAWNSDSEKPF--DDHLQKGALLYSNNSKLTSQANSNYRI LNRTPTNQTGKKD
Gt f D	SFVKTQPNWNSQTESDTSAGEKDHQGGALLYSNSDKTAYANS DYRLLNRTPTSQTGK--
Gt f I - dn	AFVKTQPQWNGESEKPY--DDHLQNGALKFDNQSDLT PDTQSNYRLLNRTPTNQTGSLD
Gt f S	QFVKSQNQWSIASENETVYPNQDHMQGGALLFSNSKDEHANSDWRLLNRP TFQTGKQK
Gt f I - sb	AFVKTQPQWNGESEKPY--DDHLQNGALLFDNQD LTPDTQSNYRLLNRTPTNQTGSLD
Gt f T	AFVATQSRWNKDSEQYDKA--DHLQGGALLYTNNNLTEWANSNWRLNRTPTRQDGKTH
	 * . . * . * . *
Gt f J	KSILDEQSDPNHMGGFDFLLANDVDLSNPVVQAEQLNQIHYLMNWGSIVMGDKD--ANFD
Gt f K	-YIKNHKIVNGSDGGYEFLLSNDIDNSNPVVQAEMLNQLYYFMNWGQIVFGDKDKDAHFD
Gt f L	PK--YTKDTS--NGGFEFLLANDIDNSNPVQAEQLNLWLHYIMNIGTIT--GGSEDENFD
Gt f M	RK--YHIDRS--NGGYELLLANDIDNSNPVQAEQLNLWLHYIMNIGSIL--GNDPSANFD
Gt f B	PRYT--ADNT--IGGYEFLLANDVDNSNPVVQAEQLNLWLHFLMNFGNIY--ANDPDANFD
Gt f C	PRYT--ADRT--IGGYEFLLANDVDNSNPVVQAEQLNLWLHFLMNFGNIY--ANDPDANFD
Gt f D	PK--YFEDNS--SGGYDFLLANDIDNSNPVVQAEQLNLWLHYLMNYGSIV--ANDPEANFD
Gt f I - dn	SRFTYNANDP--LGGYELLLANDVDNSNPVQAEQLNLWLHYLLNFGTIY--AKDADANFD
Gt f S	---YFT-TN--YAGYELLLANDVDNSNPVVQAEQLNLHLYLMNWGDIV--MGDKDANFD
Gt f I - sb	SRFTYNPNDP--LGGYDFLLANDVDNSNPVVQAEQLNLWLHYLLNFGSIY--ANDADANFD
Gt f T	YS-----KADKYGGYEFLLANDVDNSNPVVQAEMLNQIHYLMNWGEIVMGDKN--ANFD
	. * . . . * . * . * . * . * . * . * . * . * . *

Gt f J	GIRVDAVDNVDADMLQLYTNYFREYYGVNKSEANALAHISVLEAWSLNDNHYNDKTDGAA
Gt f K	GIRVDAVDNVSVDMLQLVSSYMKAAAYKVNESARALANISILEAWSHNDPYYVNEHNTAA
Gt f L	GVRVDAVDNVNADLLQIASDYFKAKYGADQSQDQAIKHLISILEAWSHNDAYYNEDTKGAQ
Gt f M	GVRIDAVDNVDADLLQIASDYFKEKYRVADNEANAIAHLISILEAWSYNHDQYNKDTKGAQ
Gt f B	SIRVDAVDNVDADLLQIAGDYLKAAKGIIHKNDKAANDHLSILEAWSNDNTPYLHDDGDNM
Gt f C	SIRVDAVDNVDADLLQIAGDYLKAAKGIIHKNDKAANDHLSILEAWSYNTPYLHDDGDNM
Gt f D	GVRVDAVDNVNADLLQIASDYLKAHYGVKSEKNAINHLSILEAWSNDNPQYNKDTKGAQ
Gt f I - dn	SIRVDAVDNVDADLLQISSDYLKAAYGIDKNNKNANNHVSIVEAWSNDNTPYLHDDGDNL
Gt f S	GVRVDAVDNVNADLLQIQRDYKAKYGTQNEKNAIDHLSILEAWSGNDNDYVKDQNNFS
Gt f I - sb	SIRVDAEDNVDADQLQISSDYLKAAYGIDKNNKNANNHVSIVEAWSNDNTPYLHDDGDNL
Gt f T	GIRVDAVDNVNADTLQLYTNYFNSVYGVNKSEAQALAHISVLEAWSYNNDNDYNQDTNGAA . . * * * * *
Gt f J	LAMENKQRLALLFSLAKPIKERTPAVSPLYNNTFNTTQRDEKTDWINKDGSKAYNEDGTV
Gt f K	LSMDNGLRLSIVHGLTRPVTNKGTG-----ARNASMKDLING-----GYF--GLS
Gt f L	LPMDDEPMHLALVYSLLRPIGN-----RSGVEPLIS-----NSLN
Gt f M	LSIDNPLRETLTTLTFLR-----KSNY-----RGSLEPVIT-----NSLN
Gt f B	INMDNKLRLSLLFSLAKPLNQ-----RSGMNPLIT-----NSLV
Gt f C	INMDNRLRLSLLYSLAKPLNQ-----RSGMNPLIT-----NSLV
Gt f D	LPIDNKLRLSLLYALTRPLEKDas-----NKNEI-----RSGLEPVIT-----NSLN
Gt f I - dn	MNMDNKFRLSMLWSLAKPLDK-----RSGLNPLIH-----NSLV
Gt f S	LSIDNDQRSGLKAF-----G-----YASAY-----RGNLSNLAT-----AGLK
Gt f I - sb	MNMDNKFRLSMLWSLAKPTDV-----RSGLNPLIH-----NSLV
Gt f T	LAMDNGLRFSLLYTLTRPINERTPGMSTLIKSEYGLTDRTK-----
Gt f J	KQSTIGKYNEKYGDASGNYVFIRAHDNVQDIIAEI IKKEI -NPKSDGFT -ITDAEMKQA
Gt f K	NRAEVTSYDQL---GFATYLFVRAHDSEVQTVIADIISKKI -DPTTDGFT -FTLDQLKQA
Gt f L	DRSE---SGKNSKRMANAFVRAHDSEVQSIIGQIIKNEI -NPQSTGNT -FTLDEMCKA
Gt f M	NRSS---EQKHTPRDANYIFVRAHDSEVQAVLANIISKQI -NPKTDGFT -FTMDELKQA
Gt f B	NRTD---DNAETAAPVPSYSFIRAHDSVQDLIADI IKA EI -NPNVVGYS -FTMEEIKKA
Gt f C	NRTD---DNAETAAPVPSYSFIRAHDSVQDLIRNIIRTEI -NPNVVGYS -FTTEEIKKA
Gt f D	NRSA---EGKNSERMANYIFIRAHDSVQTVIAKIIKAQI -NPKTDGLT -FTLDELKQA
Gt f I - dn	DREV---DDREVETVPSYSFARAHDSEVQDLIRDI IKA EI -NPNAFGYS -FTQDEIDQA
Gt f S	NRSA---NP-DSDPVPNYVFIRAHDSVQTRI AKI IREKLGKTNADGLTNLTLDLNLKA
Gt f I - sb	DREV---DDREVETVPSYSFARAHDSEVQDIIRDIIKA EI -NPNSFGYS -FTQEEIDQA
Gt f T	-----NDKYGDTQPSYVFVRAHDSEVQTVIAQIIKEKI -DPTTDGFT -FTLDQLKQA * * * * * * *
Gt f J	FEIYNKDMLSDDKKYTLNNI PAAYAVMLQNMETITRVYYGDLYTDDGHYMETKSPYYDTI
Gt f K	FDIYNADMLKVDKEYTHSNI PAAYALMLQTMGAATR VYYGDLYTDNGQYMAKKS PYFDQI
Gt f L	FEIYNKDMRSANKQYTQYNI PSAYALMLTHKDTVPRVYYGDMYTDDGQYMAQKSPYYDAI
Gt f M	FEIYNADIAKADKKYTQYNI PAAYATMLTNKDSITRVYYGDLFTDDGQYMAEKSPYYNAI
Gt f B	FEIYNKDLLATEKKYTHYNTALS YALLLTNKSSVPRVYYGDMFTDDGQYMAHKTINYEAI
Gt f C	FEIYNKDLLATEKKYTHYNTALS YALLLTNKSSVPRVYYGDMFTDDGQYMAHKTINYEAI
Gt f D	FKIYNEDMRQAKKKYTQSNIP TAYALMLSNKDSITRLYYGDMYSDDGQYMATKSPYYDAI
Gt f I - dn	FKIYNEDLKKTDKKYTHYNVPLSYTLLLTNKGSI PRVYYGDMFTDDGQYMAKNTVNYDAI
Gt f S	FDIYNQDMNATDKVYYPNNLPMAYAWMLQNKDTVTRVYYGDMYTDDGQYMATKTPFYNAI
Gt f I - sb	FKIYNEDLKKSDKKYTHYNVPLSYTLLLTNKGSI PRVYYGDMFTDDGQYMAKNTVNYDAI
Gt f T	FEIYNKDMNSVKNHYTHYNI PAAYAVMLSNMESVTRVYYGDLFTDDGQYMASKSPYYDAI * * * * * * *
Gt f J	VNLMKSRIKYVSGGQ--AQRSYWLPTDGKMDNSDV -ELYRTNEVYTSVRYGKDIMTANDT
Gt f K	TTLLKARPKYVAGGQ---TSY---IHNLAGDGVSSAKDNKEVLVSVRYGQDLMSTDT
Gt f L	ETLLKGRIRYAAGGQDMKVN-----YIGYGNTN -GWDAGVLT SVRYGTGANSASDT
Gt f M	DALLRARIKYVAGGQDMKVT-----KLNG-----YE -IMSSVRYGKGAEANQL
Gt f B	ETLLKARIKYVSGGQAMRNQ-----QVGNSE-----IITSVRYGKGALKATDT
Gt f C	ETLLKARIKYVSGGQAMRNQ-----QVGNSE-----IITSVRYGKGALKATDT
Gt f D	DTLLKARIKYAAGGQDMKIT-----YVEGDKSHMDWDYTGVLT SVRYGTGANEATDQ
Gt f I - dn	ESLLKARMKYVAGGQAMQNY-----QIGNGE-----ILTSVRYGKGALKQSDK
Gt f S	ETLLKGRIKYVAGGQAVSYK-----Q-----DWS -SGILTSVRYGKGANSASDA
Gt f I - sb	ESLLKARMKYVAGGQAMQNY-----QIGNGE-----ILTSVRYGKGALKQSDK
Gt f T	NTLLRARIRYAAGGQIMEHNSYKPSVAMKAAHPDAGNV LGNSEVLVSVRFGQDVMSADDM * . . . * . * *

Gt fJ	EGSKYSRTSGQVTLVANNPKLNLDQSAKLNVE MGKIHANQKYRALIVGTADGIKNFTSDA
Gt fK	EGGKYGRNSGMLTLIANNPDLKLADGETITVNMGAAHKNQAYRPLLLGTEKGIVSSLNDS
Gt fL	-GTAETRNQGMVIVSNQPALRLTSN--LTINMGAAHRNQAYRPLLLTTNDGVATYLNDS
Gt fM	-GTAETRNQGMVLVTANRPDMKLGANDRLVVNMGAAHKNQAYRPLLLSKSTGLATYLNDS
Gt fB	-GDRTRRTSGVAVIEGNNPSLRLKASDRVVNMGAAHKNQAYRPLLLTTDNGIKAYHSDQ
Gt fC	-GDRTRRTSGVAVIEGNNPSLRLKASDRVVNMGAAHKNQAYRPLLLTTDNGIKAYHSDQ
Gt fD	-GSEATKTQGMVITSNNPSLKLNDKVI VNMGAAHKNQAYRPLLLTTKDG LTSYTSDA
Gt fI-dn	-GDATTRTSGVGVMGNQPNFSLDGKV-VALNMGAAHANQEYRALMVSTKDG VATYATDA
Gt fS	-GNTETRNSGMALLINNRPNFR--AYRNLTLMGAAHKSQAYRPLLLSTKDG IATYLNDS
Gt fI-sb	-GDATTRTSGVGVMGNQPNFSLDGKV-VALNMGAAHANQEYRALMVSTKDG VATYATDA
Gt fT	TGGKLAKTSGMFTLISNNPELELDVNEEIKVNVGKIHAGQAYRPLLLTTDKGLQKYLND
	* . . . * . . . * . . . * . . . *
Gt fJ	DAIAAGYVKETDSNGVLTFGANDIKGYETFDMSGFVAVWVPVGASDNQDIRVAPSTEAKK
Gt fK	DTK---IVKYTDAQGNLVFTADEIKGFKTVDMSGYLSVWVPVGATDDQNVLAKPSTKAYK
Gt fL	D-AN-GIVKYTDGNGNLTFANEIRGIRNPQVDGYLAVWVPVGASENQDVRVAPSKEKNS
Gt fM	DVPA-GLVRYTDNQGNLTFTADDIAGHSTVEVSGYLAVWVPVGASENQDARTKASSTKKG
Gt fB	EA--AGLVRYTNDRGELIFTAADIKGYANPQVSGYLGWVPVGAALIKMFALRLAR-PHQ
Gt fC	EA--AGLVRYTNDRGELIFTAADIKGYANPQVSGYLGWVPVGAADQDVRVAASTAPST
Gt fD	A-AK-SLYRKTNDKGELVFDASDIQGYLNPQVSGYLGWVPVGASDNQDVRVAASNKANA
Gt fI-dn	DASKAGLVKRTDENG YLYFLNDDLKGVANPQVSGFLQVWVPVGAADDQDIRVAASDTAST
Gt fS	DV-DSRQYKYTDSQGNLSFSASELQSVANAQVSGMIQVWVPVGAADNQDVRTSPSTQATK
Gt fI-sb	DASKAGLVKRTDENG YLYFLNDDLKGVANPQVSGFLQVWVPVGAADDQDIRVAASDTAST
Gt fT	DTK---LTKVADKDGFITFKGSEIKGYKQVEVNGYLSVWVPVGAADQDIRVAPSTAAGK
	. . . * . * . . . * . . . *
Gt fJ	EGELTLKATEAYDSQLIYEGFSNFQTI PDGSDPSVYTNRKIAENVDLFKSWGVTSFEMAP
Gt fK	EGDKVYSSSAALEAQVIYEGFSNFQDFVK--EDSQYTNKLIANADLFKSWGITSFEIAP
Gt fL	SGL-VYESNAALDSQVIYEGFSNFQDFVQ--NPSQYTNKKIAENANLFKSWGITSFEFAP
Gt fM	E-Q-VFESSAALDSQVIYEGFSNFQDFVK--TPSQYTNRVIAQNAKLFEWGITSFEFAP
Gt fB	QMA-SVHQNAALDSRVMFEGFSNFQAFAT--KKEEYTNVVIKNDVKFAEWGVTDFEMAP
Gt fC	DGK-SVHQNAALDSRVMFEGFSNFQAFAT--KKEEYTNVVIKNDVKFAEWGVTDFEMAP
Gt fD	TGQ-VYESSAALDSQLIYEGFSNFQDFVT--KDSYTNKKIAQNVQLFKSWGVTSFEMAP
Gt fI-dn	DGK-SLHQDAAMDSRVMFEGFSNFQSFAT--KEEYTNVVIANNVDK FVSWGITDFEMAP
Gt fS	DGN-IYHQSDALDSQVIYEGFSNFQAFQAQ--SPDQYTNNAVIKNGDLFKSWGITQFEMAP
Gt fI-sb	DGK-SLHQDAAMDSRVMFEGFSNFQSFAT--KEEYTNVVIANNVDK FVSWGITDFEMAP
Gt fT	EKD KTYTASQALESQLIYEGFSNFQDFVQ--KDSQYTNKKIAENTDLFKAWGVTSFEMAP
	* ***** *** * * * * * *
Gt fJ	QFVSADDGTFLDSVIQNGYAFADRYDLAMSKNNKYGSKEDLRDALKALHKAGIQAIADWV
Gt fK	QYVSSKDGTFLDSIIENGYAFTDRYDFAMSKNNKYGSKEDLRDALKALHKQGIQVIADWV
Gt fL	QYVSSDDGSFLDSVIQNGYAFTRDYDIGMSKDNKYGSLADLKAALKSLHAVGISAIADWV
Gt fM	QYVSSQDGTFLDSIIENGYAFEDRYDIAMSKNNKYGSLKDLMDALRALHAEGISAIADWV
Gt fB	QYVSSTDGSFLDSVIQNGYAFTRDYDLGISKPNKYGTADDLVKAIKALHSGIKVMADWV
Gt fC	QYVSSTDGSFLDSVIQNGYAFTRDYDLGISKPNKYGTADDLVKAIKALHSGIKVMADWV
Gt fD	QYVSSSEDGSFLDSIIQNGYAFEDRYDLAMSKNNKYGSQQDMINAVKALHSGIQVIADWV
Gt fI-dn	QYVSSTDGQFLDSVIQNGYAFTRDYDLGMSKANKYGTADQLVKAIKALHAKGLKVMADWV
Gt fS	QYVSSSEDGTFLDSVILNGYAFSDRYDLAMSKNNKYGSKQDLANAIKGLQSAGIKVLSDLV
Gt fI-sb	QYVSSTDGQFQDSVIQNGYAFTRDYDLGMSKANKYGTADQLVKAIKALHAKGLKVMADWV
Gt fT	QYVSATDGTFLDSIIENGYAFTDRYDLAMSKNNKYGSKEDLANALKALHAAGIQAIADWV
	* *
Gt fJ	PDQIYQLPGKEVVTATR TDGAGRKIADAIIDHSLYVANSKSSGKDYQAKYGGEF LAELKA
Gt fK	PDQLYTLPGKEVVTATR TDTHGKVLDDTSLVNKLYVTNTKSSGNDFQAQYGGAF LDKLQK
Gt fL	PDQIYNLPGDEVVTATRVNNYGETKDGAIIDHSLYAAKTRTFGNDYQGYGGAF LDELKR
Gt fM	PDQIYNLPGKEVVTASRTNSYGTTPRPNAEIYNSLYAAKTRTFGNDYQGYGGAF LDELKA
Gt fB	PDQMYAFPEKEVVTATRV D KYGTPVAGSQIKNTLYVVDGKSSGKDQQA KYGGAFLEELQA
Gt fC	PDQMYALPEKEVVTATRV D KYGTPVAGSQIKNTLYVVDGKSSGKDQQA KYGGAFLEELQA
Gt fD	PDQIYNLPGKEVVTATRVNDYGEYRKDSEIKNTLYAANTKSNGKDYQAKYGGAF LSELAA
Gt fI-dn	PDQMYTFPKQEVVTVTRTDKFGKPIAGSQINHSLYVTDTKSSGDDYQAKYGGAF LDELKE
Gt fS	PNQLYNLPGKEVVTATRVNQYQAKSGATINKTPYVANTRSYG-DYQE QYGGKFLDDLQK
Gt fI-sb	PDQMYTFPKQEVVTVTRTDKFGKPIAGSQINHSLYVTDTKSSGDDYQAKYGGAF LDELKE
Gt fT	PDQIYQLPGKEVVTASRV D NYGRVKVDQPLVEKLYLANTKSSGKDFQA KYGGEF LAELQK
	* *

GtfJ	KYPEMFKVMISTGKPIDDSVKLKQWKAIFYNGTNVLERGVGYVLS
GtfK	LYPEIFKEVMEASGKTIDPSVKIKQWEAKIFYNGTNIQKRGSDYVLS
GtfL	LYPQIFDRVQISTGKRMTTDEKITQWSAKYMNGTNILDRGSEYVLK-
GtfM	KYPAIFERVQISNGRKLTTNEKITQWSAKIFYNGSNIQGTGARYVLQD
GtfB	KYPELFARKQISTGVPMDPSPVKIKQWSAKIFYNGTNILGRGAGYVLKD
GtfC	KYPELFARKQISTGVPMDPSPVKIKQWSAKIFYNGTNILGRGAGYVLKD
GtfD	KYPSIFNRTQISNGKKIDPSEKITAWKAKIFYNGTNILGRGVGYVLKD
GtfI-dn	KYPELFTKKQISTGQAIDPSVKIKQWSAKIFYNGSNILGRGADYVLS
GtfS	LYPRLFSTKQISTGKPIDPSVKITNWSAKIFYNGSNILGRGAKYVLSE
GtfI-sb	KYPELFTKKQMSTGQAIDPSVKIKQWSAKIFYNGSNILGRGADYVLS
GtfT	KYPEMFTTKMISTGKTIDPSVKLKEWSAKIFYNGTNVLDRGTDYILSD
	** . * . * . * * * * . * . *

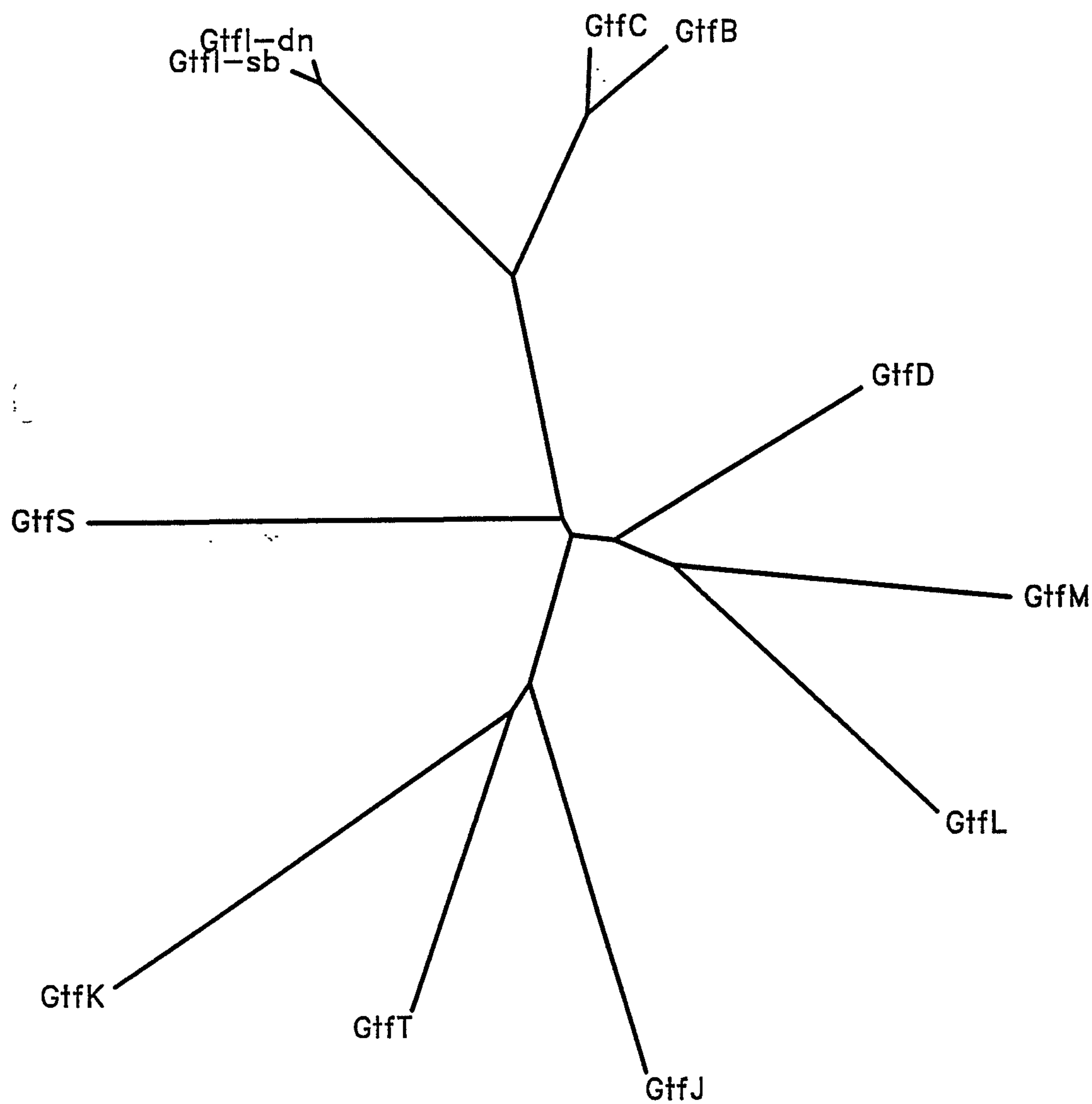


Fig. 6.12. Phylogenetic tree constructed from the multiple alignment of the central catalytic region of the 11 available Gtfs.

CHAPTER 7

¹³C-NMR ANALYSIS OF THE GLUCANS PRODUCED BY THE GLUCOSYLTRANSFERASES OF *S. SALIVARIUS*

7.1 INTRODUCTION

All streptococcal glucans are composed of linked α -D-glucosyl residues with the types of linkages determining the physical properties of the glucan (reviewed by Walker, 1978). Many studies have looked at the structure of total glucan produced by streptococci or the products of two enzyme fractions, those producing water-insoluble and those producing water-soluble glucans. The α -1,6-linked soluble glucans produced by GTF-S are thought to act as primers for the GTF-Is which synthesize chains of linear α -1,3-linked glucosyl residues onto the α -1,6-linked backbone. The result is a mixed linkage glucan that contains sequences of both α -1,6- and α -1,3-linked glucosyl residues. This mixed linkage glucan is insoluble and possesses adhesive properties. More recent work, however, has indicated that many oral streptococci produce more than two GTFs. GTF activities from streptococcal culture fluid, however, are difficult to separate as they form aggregates, proteolytically degrade, and in some cases give low yields.

Two groups working on *S. sobrinus* have successfully separated four GTF activities from this species and have studied them extensively (Takehara, 1984; Fukei *et al.*, 1982; Shimamura *et al.*, 1982; Hanada and Takehara, 1987; Yamashita *et al.*, 1988; Yamashita *et al.*, 1989; Taylor *et al.*, 1990; Walker *et al.*, 1990; Hanada and Takehara, 1991; Hanada *et al.*, 1993). The glucans produced by these enzymes have been analysed. The primer dependent GTF-I produces a glucan with mainly α -1,3-linked glucosyl residues. The other three activities produce water-soluble products of different structure.

GTFs of other oral streptococci, however, have not been as well characterised. The cloning of individual activities from these oral streptococci has allowed some structural determination of the recombinant glucan products. Little is known about the structures of glucans produced by *S. salivarius*.

7.2 METHODS AND MATERIALS

7.2.1 Partial Purification of Recombinant Glucosyltransferases

Two litre λ -lysates were prepared from each of the clones exhibiting GTF activity, i.e. λ A-8, λ A-33, λ D-10, and λ C-13. Cell debris was removed by centrifugation (5000g, 15min, 4°C). The supernatants were concentrated approximately 4-15 fold using a hollow fibre system (Amicon), and sodium azide added to 0.05% (w/v). All solutions after this point included 0.05% (w/v) sodium azide. The concentrates were dialysed overnight against 10mM potassium phosphate buffer (pH 6.5) at 4°C, centrifuged (5000g, 15 min, 4°C) to remove particulate matter and applied to an hydroxylapatite (Biorad Gel HTP) column (27cm x 1.6cm diameter) at 4°C and at a flow rate of 8ml/h. The column was washed with 3 volumes of 10mM potassium phosphate buffer (pH 6.5), before a gradient of 10mM to 300mM potassium phosphate buffer (pH 6.5) (total volume 200ml) was applied. Fractions were collected every 30min (approximately 4ml) and 50-100 μ l samples assayed for GTF activity (2.5.1; 2.5.2). Fractions with GTF activity were pooled and dialyzed against 10mM potassium phosphate buffer (pH 6.5) at 4°C overnight.

7.2.3 Preparation of Glucan

The partially purified GTF obtained by hydroxylapatite chromatography (7.2.1) was incubated at 37°C in 200ml of 10mM potassium phosphate buffer pH 6.5 containing 4% (w/v) sucrose and 0.01% (w/v) thiomersal at 37°C overnight (GtfL, GtfM) or for 5 days (GtfJ, GtfK). Glucan was also produced by GtfJ and GtfK in the presence of 0.02% (w/v) dextran T-10 by incubation of the enzymes overnight. Insoluble glucan produced by GtfJ and GtfL was collected by centrifugation and washed a number of times with H₂O. Soluble glucan produced by GtfK and GtfM was precipitated by the addition of ethanol to 75% (v/v), and recovered by centrifugation. This was followed by a series of re-precipitations by 75% (v/v) ethanol, before final resuspension in a small volume of H₂O. The glucan suspensions were frozen at -70°C, before freeze drying overnight using an Edwards Modulyo Freeze Dryer.

7.2.4 ^{13}C -NMR analysis of Glucans

^{13}C -NMR spectra were obtained at 124MHz in deuterated dimethyl sulfoxide (DMSO) solution, in a 5mm diameter sample tube, using a Bruker AM500 spectrometer operating in the Fourier-transform mode with complete proton decoupling. The probe temperature was 363°K. Spectra were run overnight accumulating 16384 data points over a spectral width of 27.7 kHz from 26 000 scans. Digital resolution was 3.39Hz/point. The internal reference was DMSO (40ppm). Assignment of peaks was based on the reports by Colson *et al.* (1974) and Gorin (1981). Spectral analyses were performed by Assoc Prof N.W.H. Cheetham at the School of Chemistry, University of New South Wales.

7.3 RESULTS AND DISCUSSION

7.3.1 Partial Purification of Recombinant Glucosyltransferase Activities

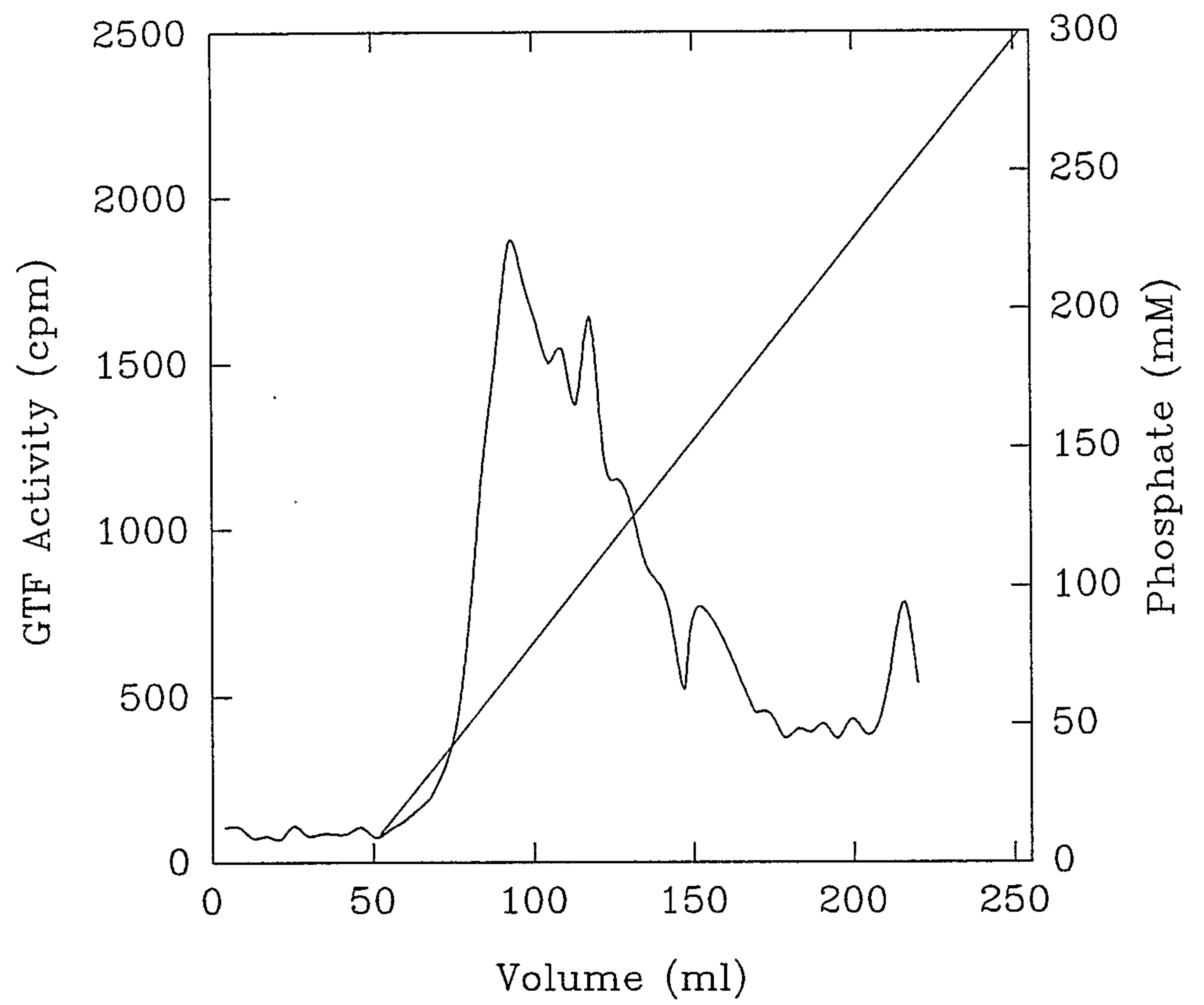
Initial ^{13}C -NMR spectra of the glucans prepared from crude λ -lysates were heavily contaminated by protein or other cellular material. Partial purification of the GTFs from λ -lysates were therefore carried out to reduce this contamination. The percentage yields of each GTF following hollow-fibre concentration and hydroxylapatite chromatography are given in Table 7.1. The recombinant GTFs were eluted from the hydroxylapatite column at phosphate concentrations between 25mM and 150mM (Fig.7.1A-D). In some cases the total GTF activity eluted from the hydroxylapatite column appeared greater than the total activity derived from the total lysate. This anomaly may be the result of the removal of inhibitory substances from the lysate, which allowed increased production of glucan. The purification procedure used did not result in pure GTFs. However, the glucans prepared from these preparations gave clear ^{13}C -NMR spectra.

7.3.2 Analysis of the Glucans Produced by *S. salivarius* Glucosyltransferases

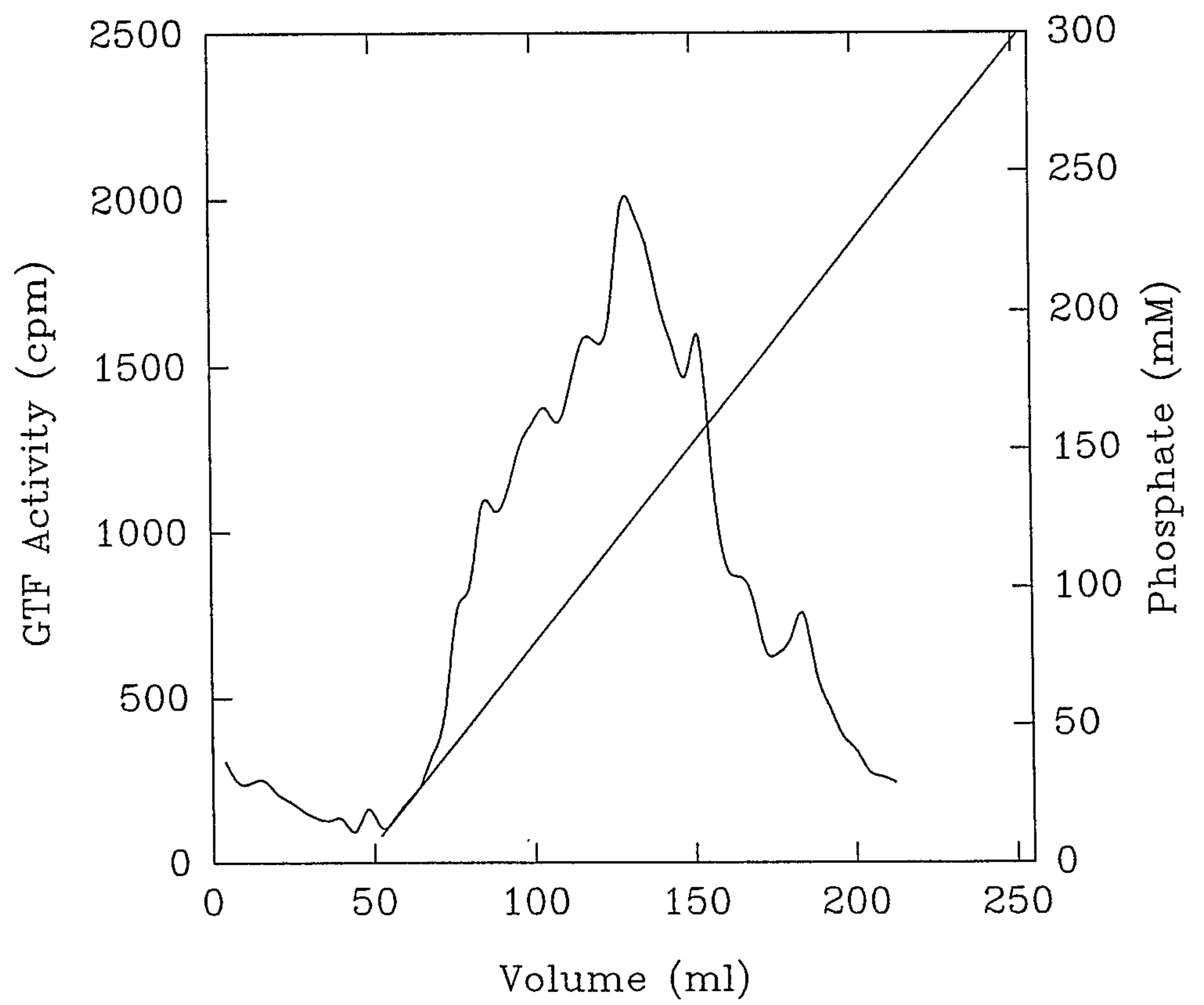
^{13}C -NMR spectroscopy allowed the determination of the major linkages within the glucans produced by the cloned GTFs of *S. salivarius* that had been partially purified from bacteriophage- λ lysates. The percentages of these linkages cannot be accurately quantified from the ^{13}C -NMR spectra and therefore the values given

Fig. 7.1. Profile of GTF activity, GtfJ (A), GtfK (B), GtfL (C) and GtfM (D), eluted from hydroxylapatite columns by a phosphate gradient.

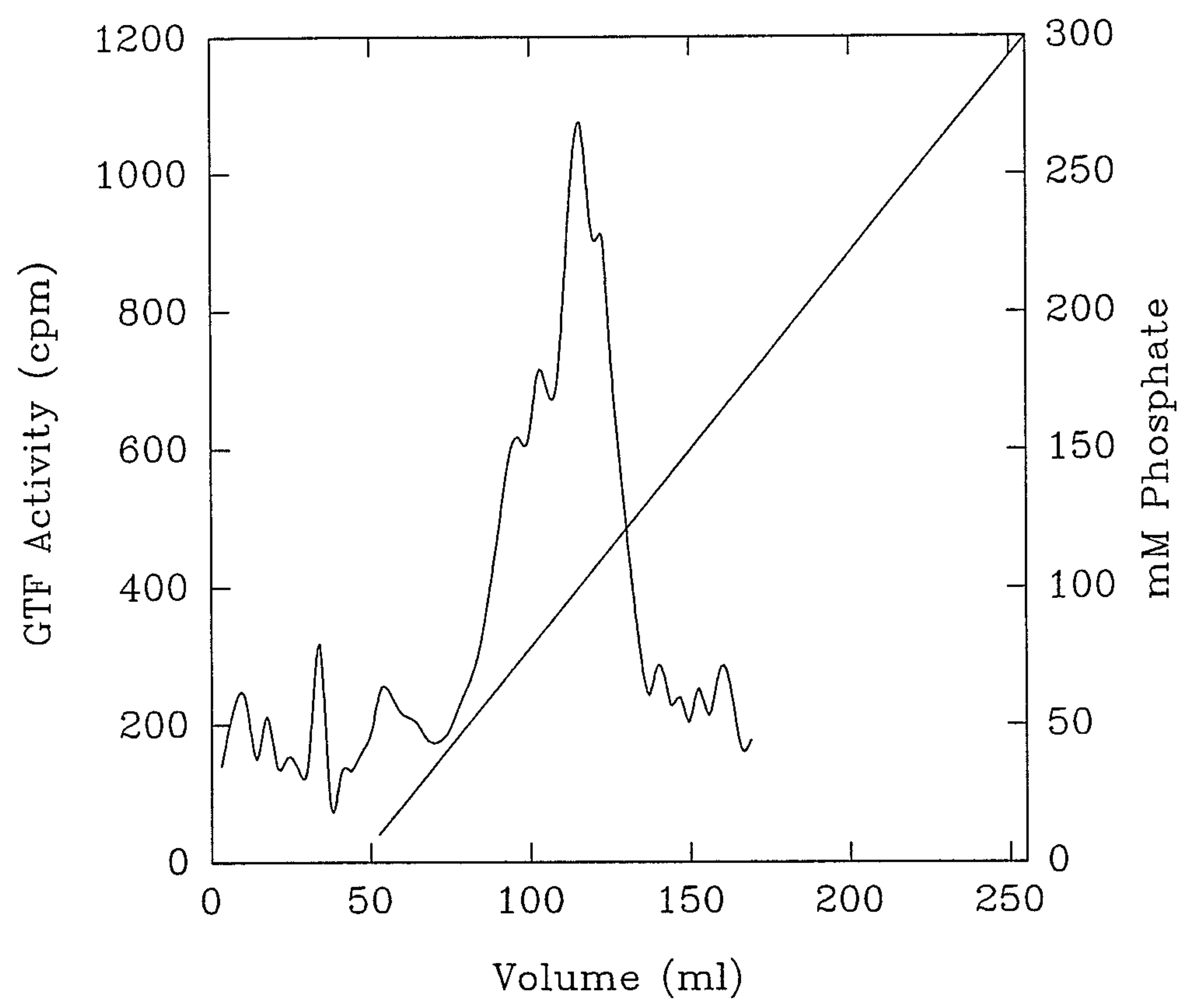
A. GtfJ



B. GtfK



C. GtfL



D. GtfM

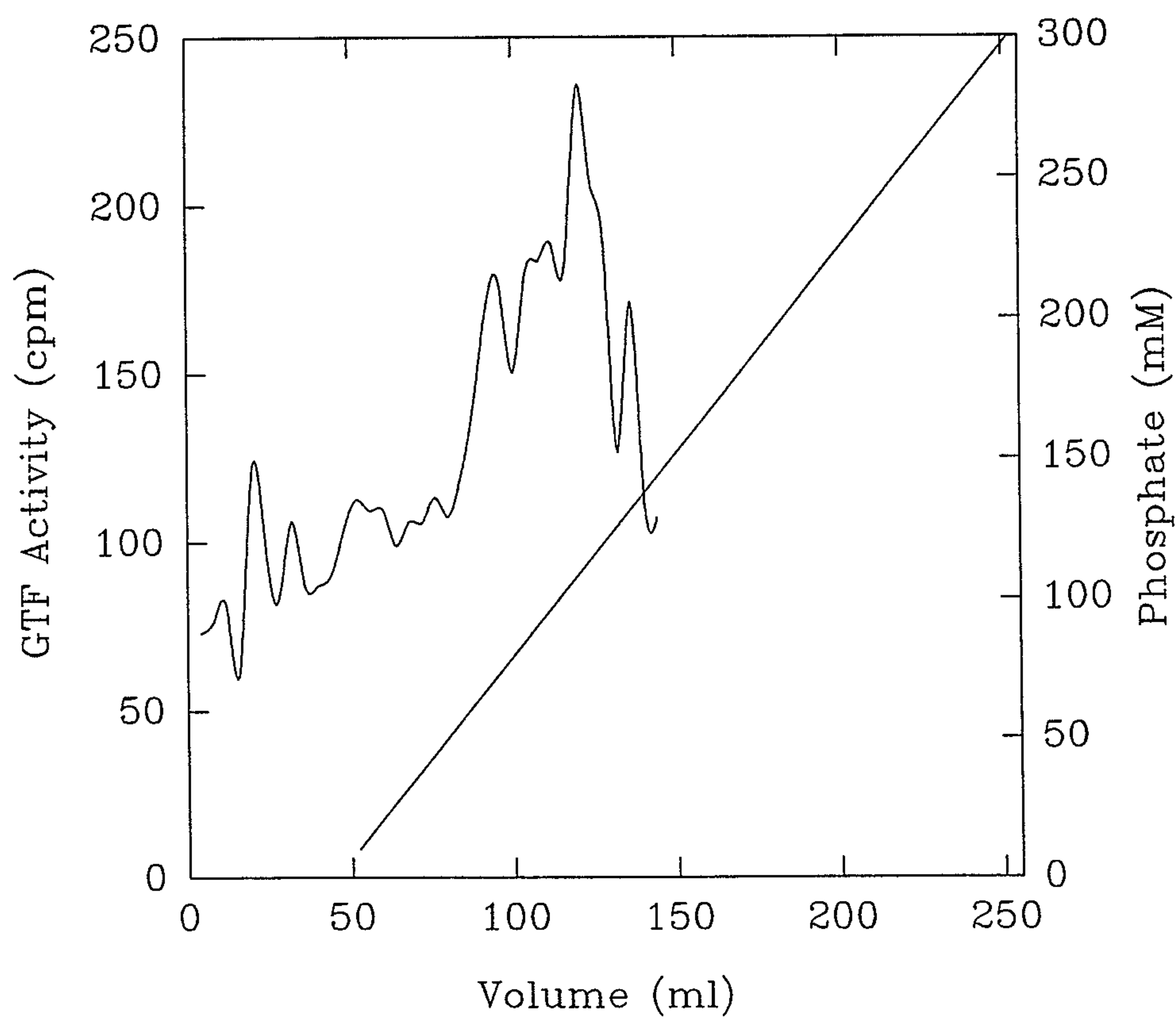


Table 7.1. Total GTF activity at each step in the concentration of bacteriophage- λ lysates and partial purification of recombinant GTFs by hydroxylapatite chromatography.

Bacteriophage	Lysate		Hollow Fibre Concentration		Hydroxylapatite	
	Volume (ml)	GTF Activity (cpm)*	Volume (ml)	GTF Activity (cpm)	Volume (ml)	GTF Activity (cpm)
λ A-8 (GtfJ)	2000	1.3×10^7 (100%)	265	7.3×10^6 (57%)	63	1.3×10^7 (102%)
λ A-33 (GtfK)	2000	9.0×10^6 (100%)	148	4.2×10^6 (47%)	101	1.5×10^7 (167%)
λ D-10 (GtfL)	2000	1.1×10^6 (100%)	200	7.4×10^5 (66%)	43	5.3×10^5 (47%)
λ C-13 (GtfM)	2000	7.1×10^5 (100%)	500	4.9×10^5 (69%)	75	3.8×10^5 (53%)

* The GTF activities of GtfJ and GtfK preparations were determined in the presence of 0.02% (w/v) dextran T-10.

below are only an estimate.

7.3.2.1 ^{13}C -NMR Analysis of Dextran T-10

Assignments of the ^{13}C -NMR signals of the six carbons in the α -1,6-glucosyl linked backbone of dextran T-10 are shown in Fig. 7.2. The signal at 61.136 is that of carbon-6 in the α -1,3-linked side chain, of which there are approximately 5% in dextran T-10.

7.3.2.2 ^{13}C -NMR Analysis of the Glucan Produced by GtfJ

The glucan produced by GtfJ in the presence of 0.02% (w/v) dextran T-10 is insoluble and composed mainly of α -1,3-linked glucosyl residues. The assignments of the six carbons in the ^{13}C -NMR spectrum are shown in Fig. 7.3A (C1'-C6'). The peaks marked C1-C6 are those of an α -1,6-linked polymer which makes up 10-15% of the total.

The spectrum for the insoluble glucan synthesized by GtfJ in the absence of dextran T-10 is dominated by the sucrose spectrum, which may indicate that little of the glucan produced by GtfJ under these conditions dissolved in DMSO (Fig. 7.4). The reason for the sucrose contamination of the sample was not immediately obvious as it was prepared in a like manner to the other samples. It may simply have been that sucrose became entrapped in the insoluble product formed by the GtfJ. G1-G6 refer to the carbons from the glucose unit of sucrose. F1-F6 refer to the carbons of the fructose unit of sucrose. The other peaks do not match α -1,6-linked (Fig. 7.2) or α -1,3-linked (Fig. 7.3) glucosyl residues, but may be consistent with an oligosaccharide structure.

The water-insoluble glucan produced by GtfJ in the presence of dextran T-10 is similar to that produced by other primer-dependent GTF-I enzymes. For instance the GTF-I of *S. sobrinus* 6714 produces glucan of 93% α -1,3-linked glucosyl residues (Fukui *et al.*, 1982) and the glucan produced by the GTF-I of *S. sobrinus* AHT is composed of 79% α -1,3-linked glucosyl residues (Hanada and Takehara, 1987). Glucans produced in the absence of primer by the GtfI of *S. downei* MFe28 expressed in *E. coli* (Russell *et al.*, 1987), and the GTF-I of *S. sobrinus* (Shimamura, 1989) consist of essentially α -1,3-linked glucosyl residues.

Fig. 7.2. ^{13}C -NMR spectrum of dextran T-10. Peaks marked C1-C6 represent the signals from the six carbons of α -1,6-linked glucosyl residues. The remaining peak, C-6', represents the carbon-6 peak of α -1,3-linked glucosyl residues.

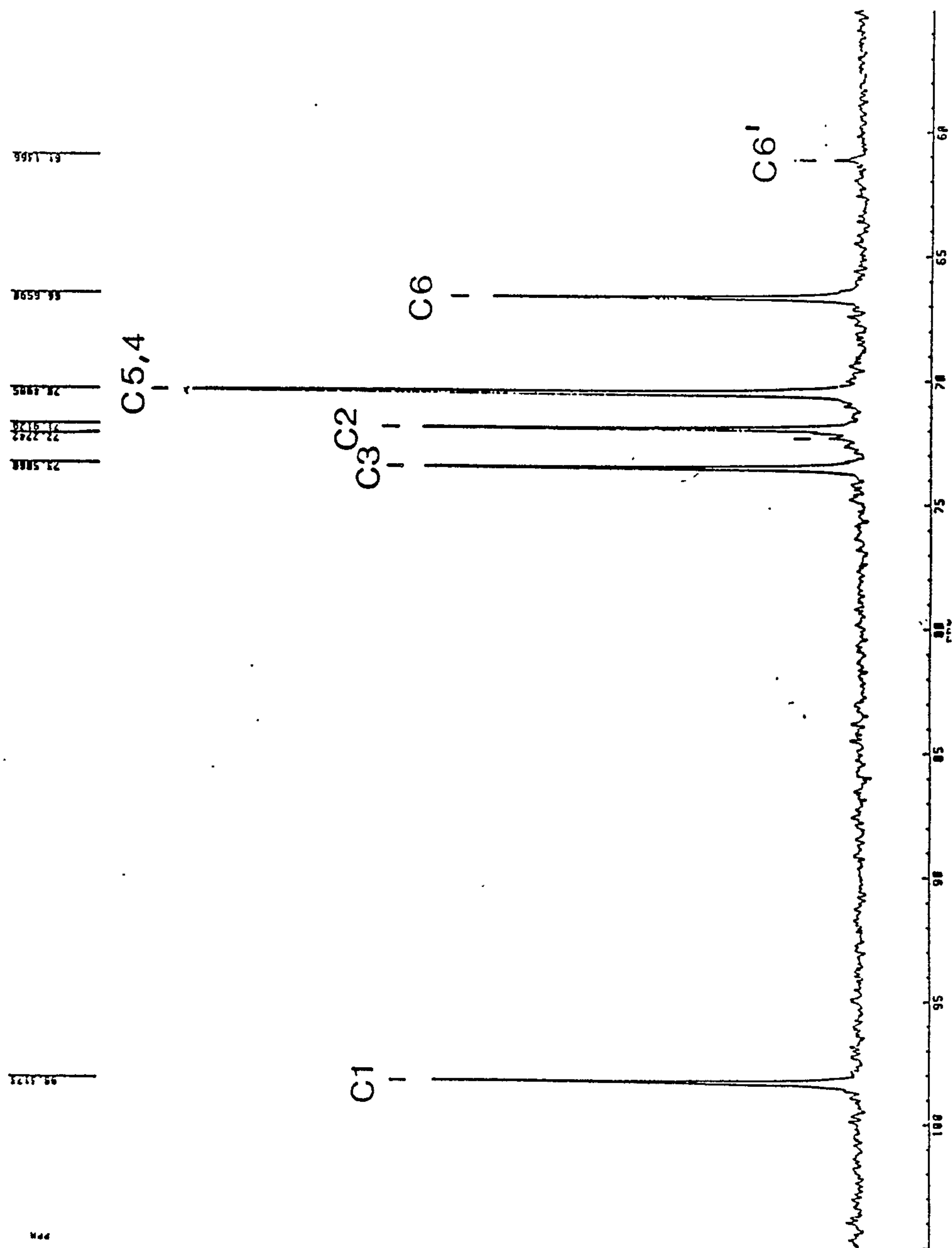


Fig. 7.3. ^{13}C -NMR spectrum of the glucan produced by GtfJ in the presence of 0.02% (w/v) dextran T-10. Peaks marked C1-C6 represent the signals from the six carbons of α -1,6-linked glucosyl residues. The peaks C1'-C6', represent the six carbons of α -1,3-linked glucosyl residues.

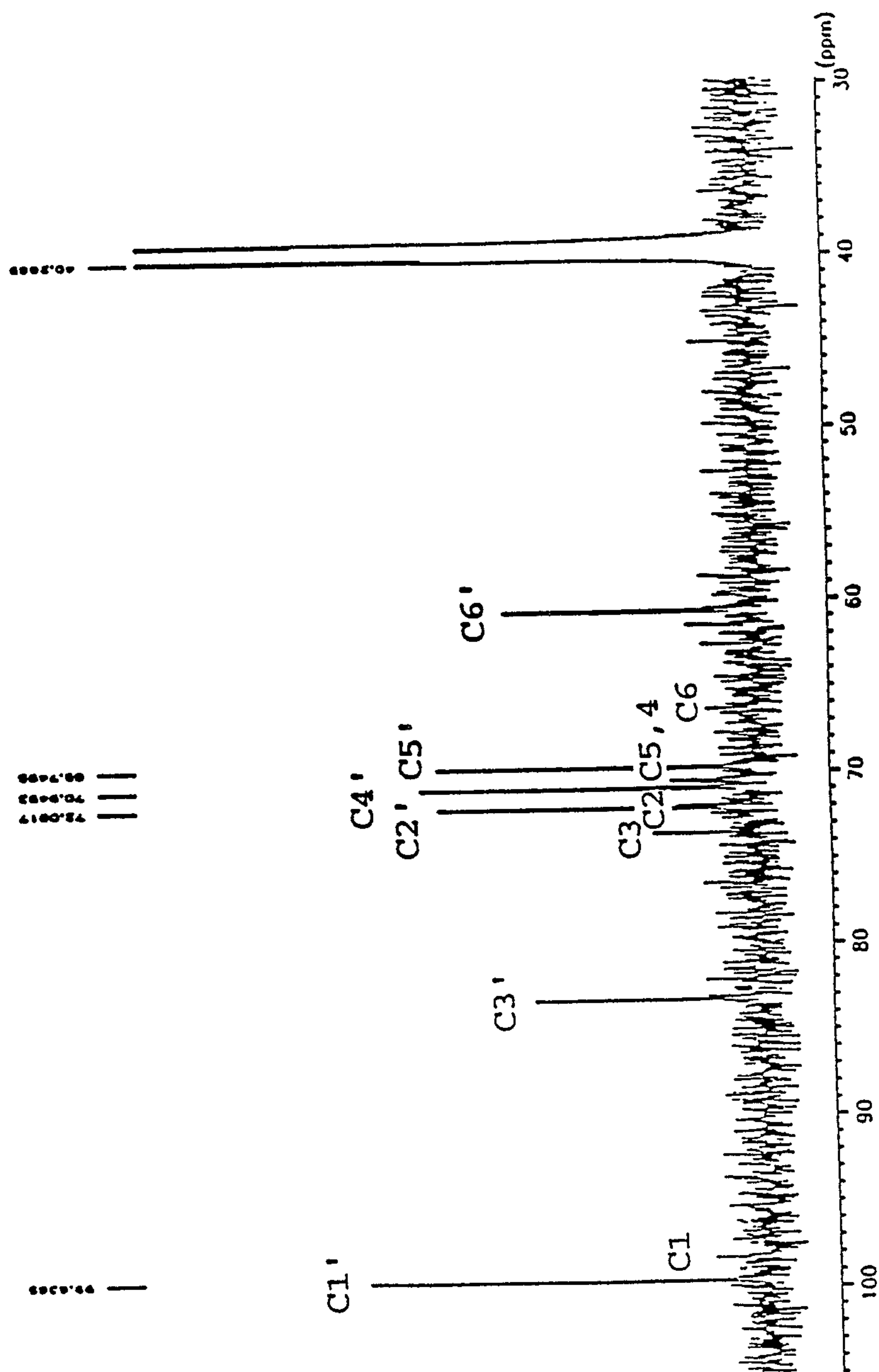
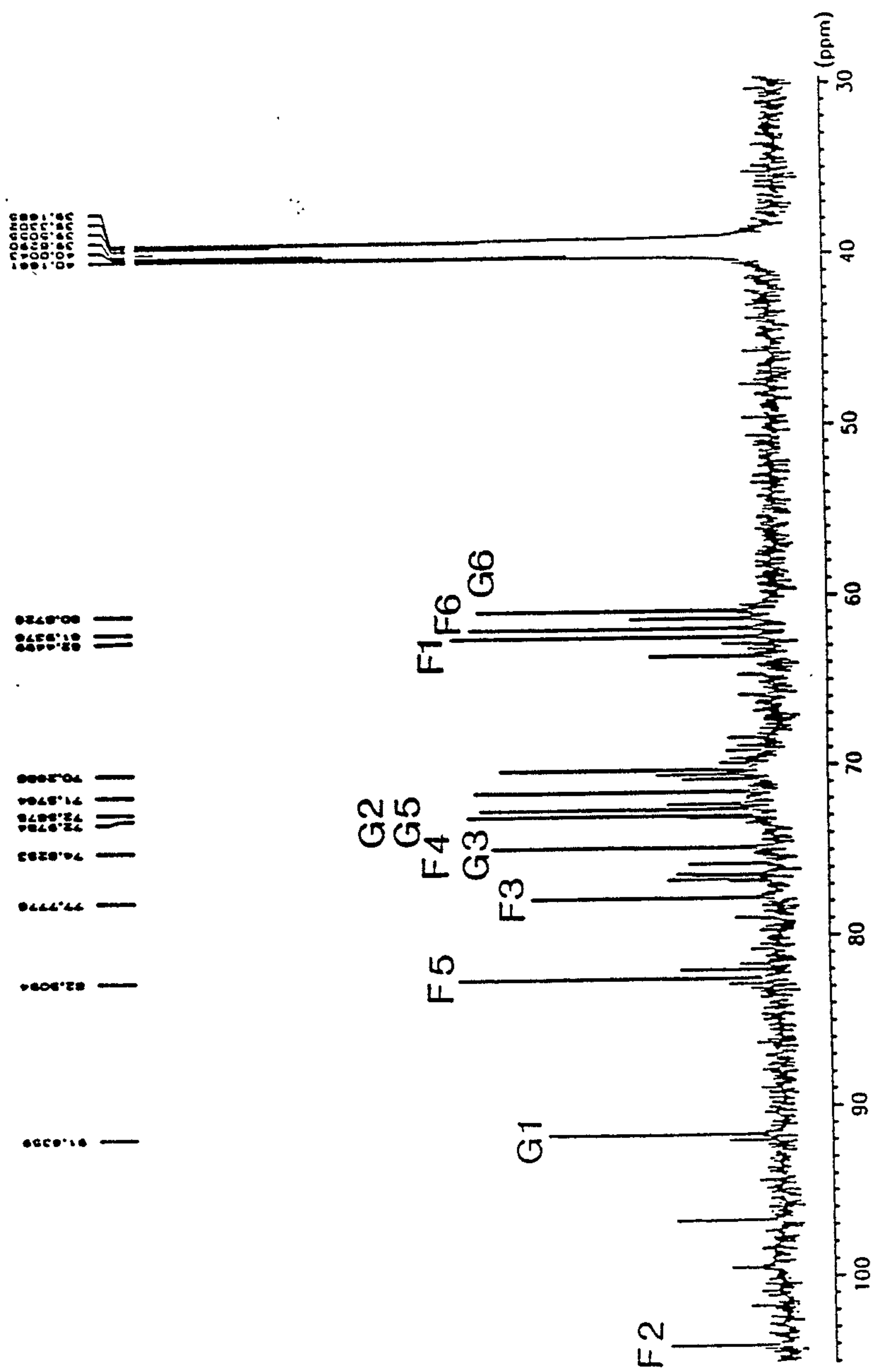


Fig. 7.4. ^{13}C -NMR spectrum of the glucan produced by GtfJ in the absence of dextran T-10. Peaks marked G1-G6 represent the signals from the six carbons of the glucose unit of sucrose. The peaks F1'-F6', represent the six carbons of the fructose unit of sucrose.



In contrast, the structure of the glucan produced by GtfJ in the absence of primer could not be determined.

7.3.2.3 ^{13}C -NMR Analysis of the Glucan Produced by GtfK

The soluble glucan produced by GtfK in the presence (Fig. 7.5) or absence of dextran T-10 (Fig. 7.6) is essentially composed of 100% α -1,6-linked glucosyl residues, with no detectable branch points. There are two resonances at 70.42 and 70.38 ppm for C4 and C6 in the ^{13}C -NMR spectrum for the glucan synthesized in the presence of dextran T-10 (Fig. 7.5), which were not resolved in the dextran T-10 spectrum (Fig. 7.2) or the spectrum for the glucan synthesized by GtfK in the absence of primer (Fig. 7.6). This indicated that the spectrum for the glucan from GtfK in the presence of dextran T-10 was of a higher resolution. The broader bases of peaks in the spectrum of the glucan synthesized without primer may indicate that the glucan has a higher molecular weight than the glucan synthesized in the presence of dextran T-10 (Fig. 7.6). Alternatively, it may be that the concentration of the sample is lower and thus the baseline noise more prominent.

The essentially α -1,6-linked structure of the glucan produced by GtfK is in contrast to the glucan produced by the primer-dependent GTF-S of *S. sobrinus* which contains 32% α -1,3-branch points (Taylor *et al.*, 1990). A primer-dependent GTF-S enzyme, GtfD, has been cloned from *S. mutans* (Hanada and Kuramitsu, 1989), however the structure of the glucan it synthesizes has not been reported.

7.3.2.4 ^{13}C -NMR Analysis of the Glucan Produced by GtfL

The glucan produced by GtfL contains both α -1,6-linked (C1-C6) and α -1,3-linked (C1'-C6') glucosyl residues (Fig. 7.7). This linkages appear to be present in roughly equal proportions. The other minor resonance at 61.09ppm is consistent with a α -1,3-branch point.

The structure of the glucan produced by GtfL is similar to that synthesized by a primer-independent GTF-I purified from a human isolate of *S. salivarius*, as this latter glucan also contains a high percentages of both α -1,6- and α -1,3-linked glucosyl residues (Sato and Inoue, 1991). The glucan produced by GtfL differed

Fig. 7.5. ^{13}C -NMR spectrum of the glucan produced by GtfK in the presence of dextran T-10. Peaks marked C1-C6 represent the signals from the six carbons of α -1,6-linked glucosyl residues.

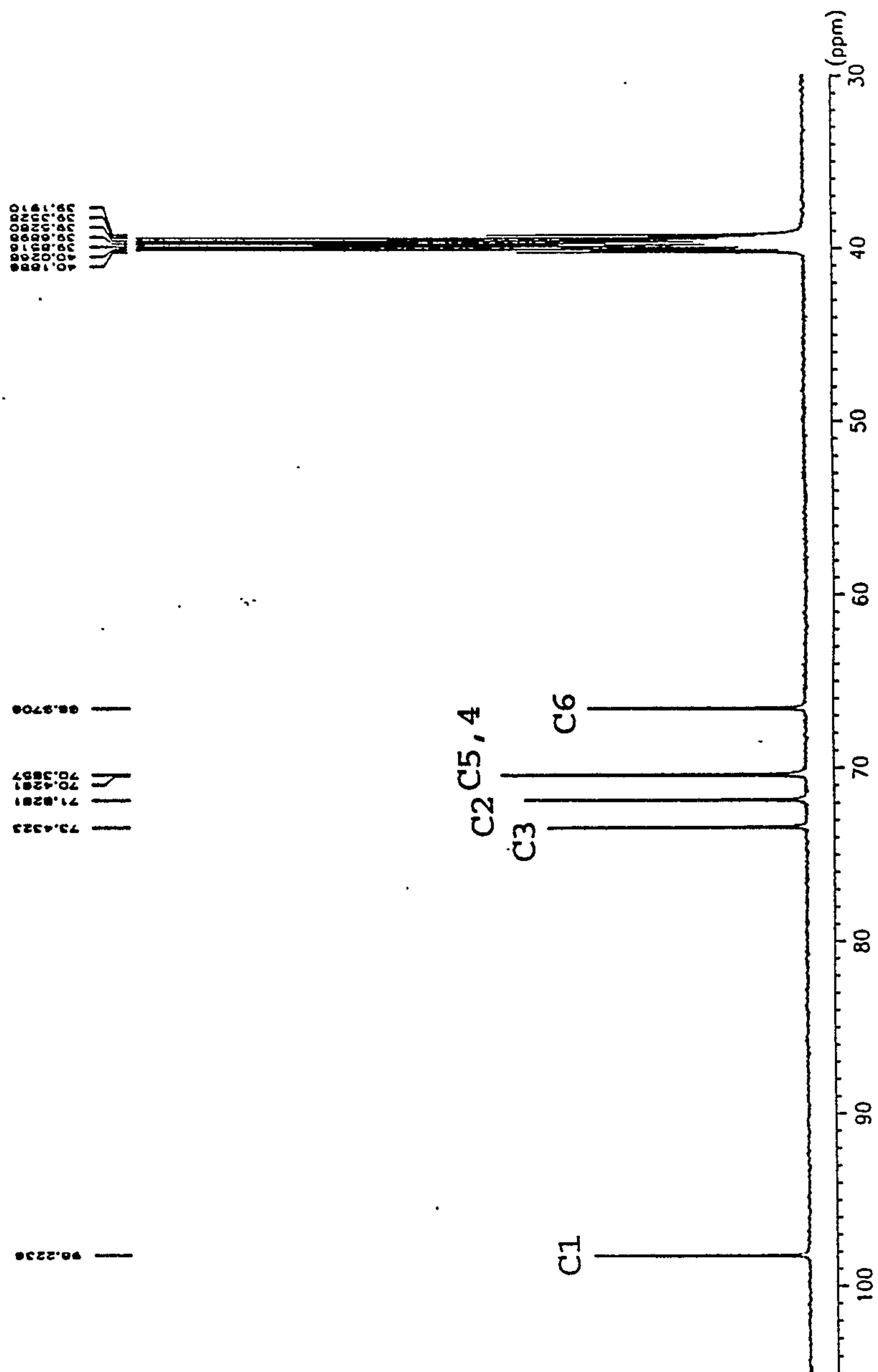
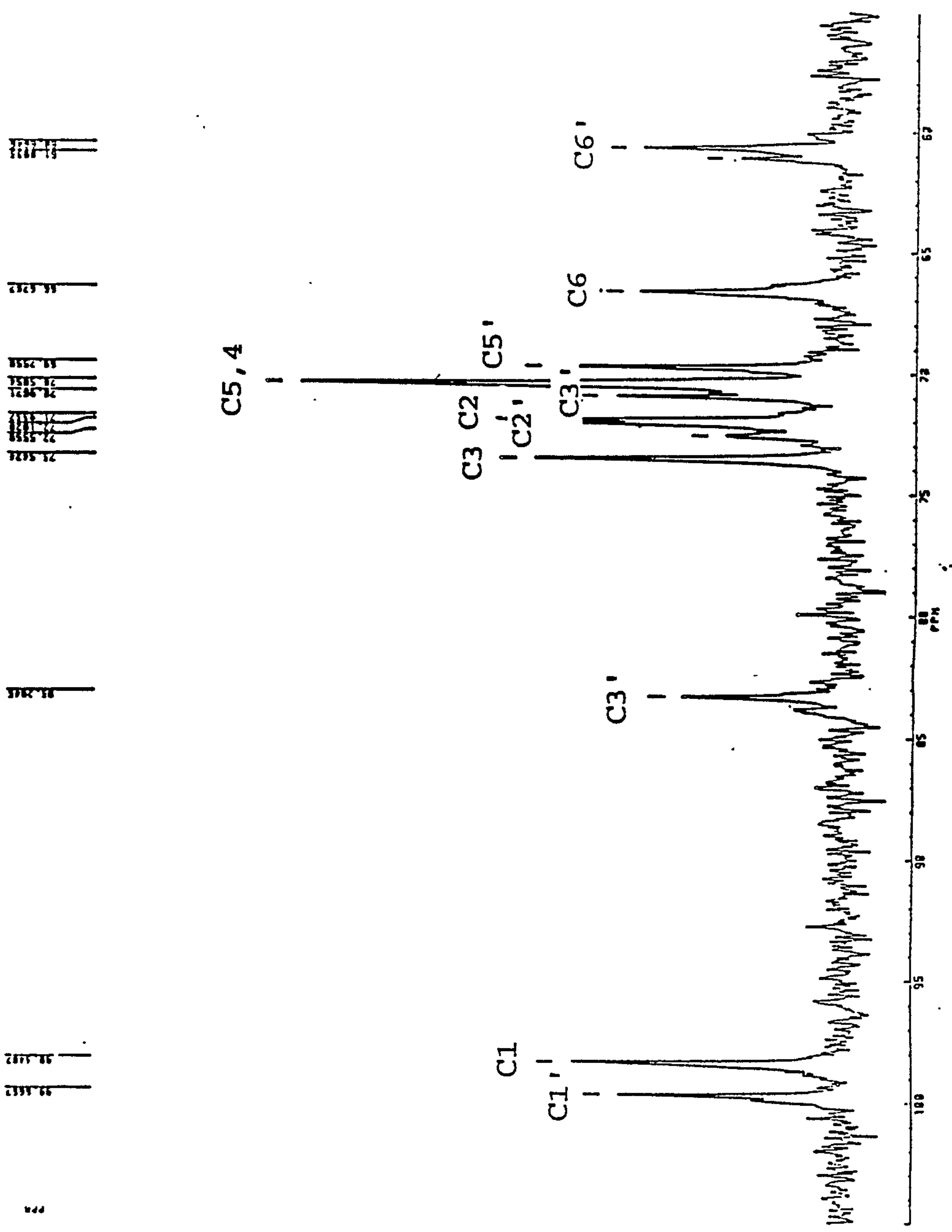


Fig. 7.6. ^{13}C -NMR spectrum of the glucan produced by GtfK in the absence of dextran T-10. Peaks marked C1-C6 represent the signals from the six carbons of α -1,6-linked glucosyl residues.



Fig. 7.7. ^{13}C -NMR spectrum of the glucan produced by GtfL. Peaks marked C1-C6 represent the signals from the six carbons of α -1,6-linked glucosyl residues. The peaks C1'-C6', represent the six carbons of α -1,3-linked glucosyl residues.

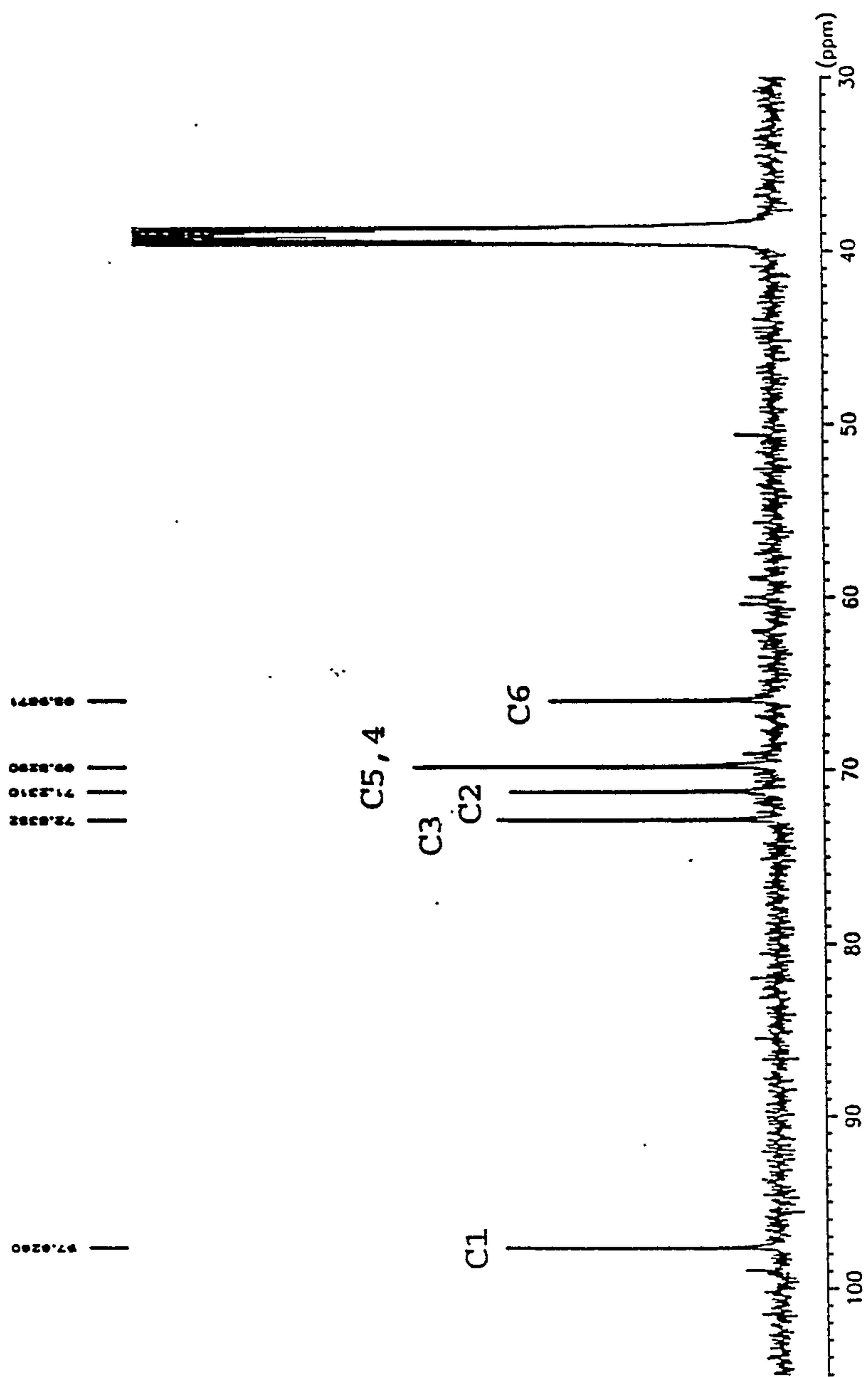


from the glucan produced in the absence of primer by the GtfB of *S. mutans*, expressed in *E. coli* as this glucan has primarily α -1,3-linked glucosyl residues (Nakano and Kuramitsu, 1992).

7.3.2.5 ^{13}C -NMR Analysis of the Glucan Produced by GtfM

The glucan produced by GtfM is essentially composed of α -1,6-linked glucosyl residues (Fig. 7.8). The minor resonances in the ^{13}C -NMR spectrum do not match those of α -1,3-linked glucosyl residues and were not assigned. Primer-independent GTF-S enzymes from *S. sobrinus* and *S. downei* are also primarily composed of α -1,6-linked glucosyl residues, with little or no branching. In each species, one of these enzymes produces linear oligo-isomaltosaccharides (Yamashita *et al.*, 1988; Taylor *et al.*, 1990; Russell *et al.*, 1990). The other primer-independent GTF-S of *S. sobrinus* produces high molecular weight α -1,6-linked glucan with 9% branch points (Taylor *et al.*, 1990). The size of the glucan produced by GtfM is currently unknown, although there is no evidence from the ^{13}C -NMR spectrum that it is composed of oligo-isomaltosaccharides.

Fig. 7.8. ^{13}C -NMR spectrum of the glucan produced by GtfM. Peaks marked C1-C6 represent the signals from the six carbons of α -1,6-linked glucosyl residues.



CHAPTER 8

KINETIC PROPERTIES OF GLUCOSYLTRANSFERASES EXPRESSED IN *ESCHERICHIA COLI*

8.1 INTRODUCTION

The expression of individual GTF activities by recombinant organisms allows these activities to be studied in some detail. The purification of GTF activities from streptococcal culture fluid can prove difficult as the enzymes may only exist at low levels, may form aggregates, may be proteolytically degraded and may bind to separation matrices. Enzymatic data from recombinant GTFs expressed in *E. coli* when compared with those from GTFs purified from streptococcal culture fluid show that the cloned and parent enzymes have the same characteristics (Russell *et al.*, 1990; Hanada *et al.*, 1991). Thus it is feasible to carry out kinetic studies using recombinant Gtf activities, rather than attempting to purify the activities from culture fluid.

GTF activities are known to differ in their pI, their optimum pH for activity, their K_m for sucrose as well as their requirement for primer and the type of glucan they produce. Analysis of the glucans produced by the four GTFs of *S. salivarius* ATCC 25975 encoded by the recombinant *gtf* genes in *E. coli* NM522 showed that they differed in structure (Section 7). The four GTFs were also known to have different requirements for exogenous glucan and to produce glucans of varying solubility (Section 5). It was therefore of interest to determine other differences between these enzymes, including their K_m values for dextran T-10 and sucrose. As glucans produced by primer-independent GTFs of *S. sobrinus* have been demonstrated to act as primers for primer-dependent GTF-S and GTF-I activities (McCabe, 1985; Yamashita *et al.*, 1989), it was also of interest to determine the acceptor efficiency of the glucans produced by the primer-independent GTFs, GtfL and GtfM of *S. salivarius*. As an activity similar to GtfL had not been previously reported its potential role in glucan synthesis was of particular interest.

8.2 METHODS AND MATERIALS

8.2.1 Preparation of Samples

E. coli NM522 transformed with phagemids expressing GTF activity (pGSG101, pGSG201, pGSG401, pGSG501) were grown at 37°C overnight (16h) in a shaking incubator in 200ml of LB medium supplemented with ampicillin (100µg/ml). Cells were harvested by centrifugation (5000g, 15min, 4°C) and resuspended in 50ml of 20mM potassium phosphate buffer (pH 6.5). The cells were sonicated on ice using a Branson Sonic Power (40W, 60s with 5-10min cooling periods for a total sonication time of 5min) and unbroken cells and cell debris removed by centrifugation (5000g, 15min, 4°C).

8.2.2 Determination of the Amount of Glucose Incorporated into Glucan

Radioactive assays (Section 2.5.2) were used to determine the amount of glucose incorporated into glucan for each GTF activity. In all experiments 1ml aliquots of assay mix were assayed in duplicate. The glucan formed in each aliquot was either precipitated by the addition of 3 volumes of ethanol and washed with 75% (v/v) ethanol, to determine the total amount of glucan synthesized, or collected on filters and washed with 10mM potassium phosphate buffer (pH 6.0) to determine the amount of insoluble glucan (Section 2.5.2). The amount of glucose incorporated into glucan was determined by the following equation:

$$\frac{X \times N}{t} = \mu\text{mole glucose incorporated/ml}$$

where X = counts of assayed material in 1ml of assay mix
 t = total counts in 1ml assayed material
 N = µmoles of sucrose in 1ml assay mix (10 unless otherwise stated)

8.2.3 Production of Glucan with Time

The amount of glucan produced at 37°C by a specific *S. salivarius* GTF expressed in *E. coli* was measured at various time intervals over a period of 6-8h. Initial experiments were carried out using a total volume of 48ml of assay mix containing 5mM sucrose and 1-10ml of sonicate, with and without the addition of 0.02%

(w/v) dextran T-10, and both insoluble and total glucan production determined (8.2.2). In later experiments 80-720 μ l of sonicate were incubated in a total volume of 24ml, with and without the inclusion of 0.02% (w/v) dextran T-10. The amount of insoluble glucan produced by the GTF-Is and the amount of soluble glucan produced by the GTF-Ss was determined (8.2.2).

8.2.4 Effect of Dextran Concentration on Glucosyltransferase Activity

Sonicates (50-100 μ l) were incubated at 37°C for 20min at pH6.0 in the presence of 10mM [^{14} C-glucosyl]-labelled sucrose and dextran T-10 over a concentration range of 0-0.4mg/ml in a total volume of 1ml. Glucans were precipitated as above (8.2.2; 8.2.3) and glucan production plotted against dextran concentration. Where dextran T-10 primed or stimulated GTF activity, Lineweaver-Burk plots were constructed.

8.2.5 Effect of Sucrose Concentration on Glucosyltransferase Activity

In order to determine the effect of sucrose concentration on the 'initial' rate of GTF activity, sonicates (50-100 μ l) were assayed at 37°C for 20min in a total volume of 1ml. The sucrose (0.25-100mM) in the assay mix was labelled with 1 or 2 μ Ci of [^{14} C-glucosyl]-labelled sucrose and the assays performed in the presence or absence of 0.02% (w/v) dextran T-10 (Section 2.5.2). The glucans were precipitated as above (8.2.2; 8.2.3). Lineweaver-Burk analyses of the data were used to calculate the K_m values for the different GTFs expressed in *E. coli*. Regression analysis was performed by the 'Sigma Plot' program (Jandel Scientific) that was also used to plot the data.

8.2.6 Effect of pH on Glucosyltransferase Activity

Assay mixes at different pH values were prepared using sodium citrate (pH 3.0, pH 4.0) and sodium phosphate buffers (pH 5.0-pH 9.0). The pH of the complete assay mix, including sucrose, was measured. Sonicates (50-200 μ l) of *E. coli* expressing GTF activity were incubated in a total volume of 1 or 2ml of assay mix for 30min at 37°C. Dextran T-10 (0.02%; w/v) was included in the assay mixes for GtfJ and GtfK. The total glucan synthesized was determined following

precipitation with ethanol (75%; v/v; 8.2.2).

8.2.6 Glucan Products as Acceptors for Primer-Dependent Activities

Non-radioactively labelled glucan was produced by GtfK, GtfL and GtfM by incubating 480-2000 μ l of a sonicate of *E. coli* cells expressing the appropriate GTF activity at 37°C for 2-6h in 48ml of assay mix in the absence of added dextran T-10. Control assays containing radioactive sucrose were run in parallel to determine the amount of glucan produced in each instance. After incubation, the samples were boiled for 10min to inactivate GTF activity. In order to minimise the fact that the glucan produced by GtfK precipitated during boiling, the sample was heated at 60°C for 10min, although some precipitation was still noted. After cooling to 37°C, sonicates of *E. coli* expressing GtfJ (160 μ l) or GtfK (480 μ l) were added, together with 0.6 μ Ci [U-¹⁴C-glucosyl]-labelled sucrose, and incubation continued at 37°C for 4h. Duplicate 1ml aliquots were assayed at various time intervals during this period to determine both insoluble and total glucan production (8.2.2).

8.3 RESULTS AND DISCUSSION

8.3.1 Preliminary Data

Initial experiments were used to determine any differences between water-insoluble and 75% (v/v) ethanol insoluble glucan produced by the individual GTFs expressed in *E. coli* NM522 over an 8h period (Tables 8.1-8.4). Experiments were initially carried out using a large volume of lysate (5-10ml) in a total volume of 48ml. Repeat experiments used different samples and a smaller volume (1-3ml) of sonicate. In all cases the glucan produced by individual enzymes was either 100% soluble or 100% insoluble. In other words there was no evidence of any soluble glucan intermediates produced by the GTF-Is, GtfJ and GtfL, or any production of insoluble glucan by the GTF-Ss, GtfK and GtfM. Consequently in all subsequent experiments, the glucans of GtfJ and GtfL were entrapped on the glass fibre discs and washed with phosphate buffer, while the glucans of GtfK and GtfM were collected by 75% (v/v) ethanol precipitation.

Table 8.1. Glucan production by GtfJ at a high and low (in parentheses) concentration of enzyme.

Time (h)	Glucan Production (μ mole glucose incorporated/ml)			
	-Dextran T-10		+Dextran T-10	
	Total	Water-Insoluble	Total	Water-Insoluble
0.25	0.2 (0.1)	0.3 (0.1)	3.7 (0.5)	3.8 (0.5)
0.5	0.2 (0.1)	0.3 (0.2)	4.3 (0.8)	4.5 (0.9)
0.75	0.2 (0.1)	0.3 (0.1)	4.3 (1.3)	4.2 (1.3)
1.0	0.2 (0.1)	0.2 (0.1)	4.2 (1.6)	4.4 (1.6)
2.0	0.2 (0.1)	0.2 (0.1)	4.2 (2.5)	4.2 (2.6)
3.0	0.2 (0.0)	0.2 (0.1)	4.3 (2.9)	4.4 (3.0)
4.0	0.2 (0.1)	0.2 (0.1)	3.5 (2.6)	4.0 (3.3)
8.0	0.0 (0.0)	0.2 (0.1)	3.4 (3.4)	4.3 (3.4)

Table 8.2. Glucan production by GtfK at a high and low (in parentheses) concentration of enzyme.

Time (h)	Glucan Production (μ mole glucose incorporated/ml)			
	-Dextran T-10		+Dextran T-10	
	Total	Water-Insoluble	Total	Water-Insoluble
0.25	0.54 (0.0)	0.0 (0.0)	2.4 (0.1)	0.0 (0.0)
0.5	0.94 (0.0)	0.0 (0.0)	3.8 (0.3)	0.0 (0.0)
0.75	1.2 (0.0)	0.0 (0.0)	4.8 (0.5)	0.0 (0.0)
1.0	1.5 (0.1)	0.0 (0.0)	4.9 (0.5)	0.0 (0.0)
2.0	2.8 (0.2)	0.0 (0.0)	5.3 (1.3)	0.0 (0.0)
3.0	- (0.3)	0.0 (0.0)	- (1.9)	0.0 (0.0)
4.0	3.2 (0.4)	0.0 (0.0)	5.1 (2.4)	0.0 (0.0)
8.0	3.8 (0.9)	0.0 (0.0)	5.2 (4.0)	0.0 (0.0)

Table 8.3. Glucan production by GtfL at a high and low (in parentheses) concentration of enzyme.

Time (h)	Glucan Production (μ mole glucose incorporated/ml)			
	-Dextran T-10		+Dextran T-10	
	Total	Water-Insoluble	Total	Water-Insoluble
0.25	0.8 (0.3)	0.7 (0.3)	0.2 (0.2)	0.2 (0.3)
0.5	0.9 (0.6)	0.9 (0.6)	0.3 (0.3)	0.2 (0.3)
0.75	0.9 (0.7)	1.0 (0.7)	0.3 (0.3)	0.2 (0.4)
1.0	0.9 (0.7)	1.0 (0.8)	0.3 (0.4)	0.2 (0.4)
2.0	0.9 (0.8)	1.0 (0.9)	0.3 (0.4)	0.2 (0.4)
3.0	0.8 (0.8)	1.0 (0.8)	0.2 (0.5)	0.2 (0.4)
4.0	0.8 (0.8)	0.9 (0.8)	0.3 (0.5)	0.2 (0.4)
8.0	0.7 (0.5)	0.9 (0.7)	0.2 (0.4)	0.2 (0.3)

Table 8.4. Glucan production by GtfM at a high and low (in parentheses) concentration of enzyme.

Time (h)	Glucan Production (μ mole glucose incorporated/ml)			
	-Dextran T-10		+Dextran T-10	
	Total	Water-Insoluble	Total	Water-Insoluble
0.25	0.8 (0.1)	0.1 (0.0)	1.1 (0.2)	0.1 (0.0)
0.5	1.4 (0.4)	0.1 (0.0)	1.7 (0.5)	0.1 (0.0)
0.75	1.9 (0.6)	0.2 (0.1)	2.2 (0.6)	0.1 (0.0)
1.0	1.9 (0.7)	0.1 (0.0)	2.5 (0.8)	0.2 (0.0)
2.0	2.6 (1.4)	0.2 (0.1)	2.7 (1.5)	0.2 (0.1)
3.0	2.2 (2.0)	0.1 (0.0)	2.7 (1.8)	0.1 (0.1)
4.0	2.4 (2.2)	0.1 (0.0)	2.6 (2.4)	0.1 (0.1)
8.0	2.4 (2.8)	0.0 (0.1)	2.5 (3.0)	0.1 (0.1)

8.3.2 Production of Glucan with Time

The rate of glucan production by individual GTFs expressed in *E. coli* were determined over a 6h period. Experiments were carried out at arbitrary (unknown) enzyme concentrations of one- and three-fold, with or without the inclusion of 0.02% (w/v) dextran T-10 (Fig. 8.1). Assaying three times the concentration of enzyme did not triple the production of glucan, except in the case of GtfK, suggesting that sucrose limitation or product inhibition was occurring.

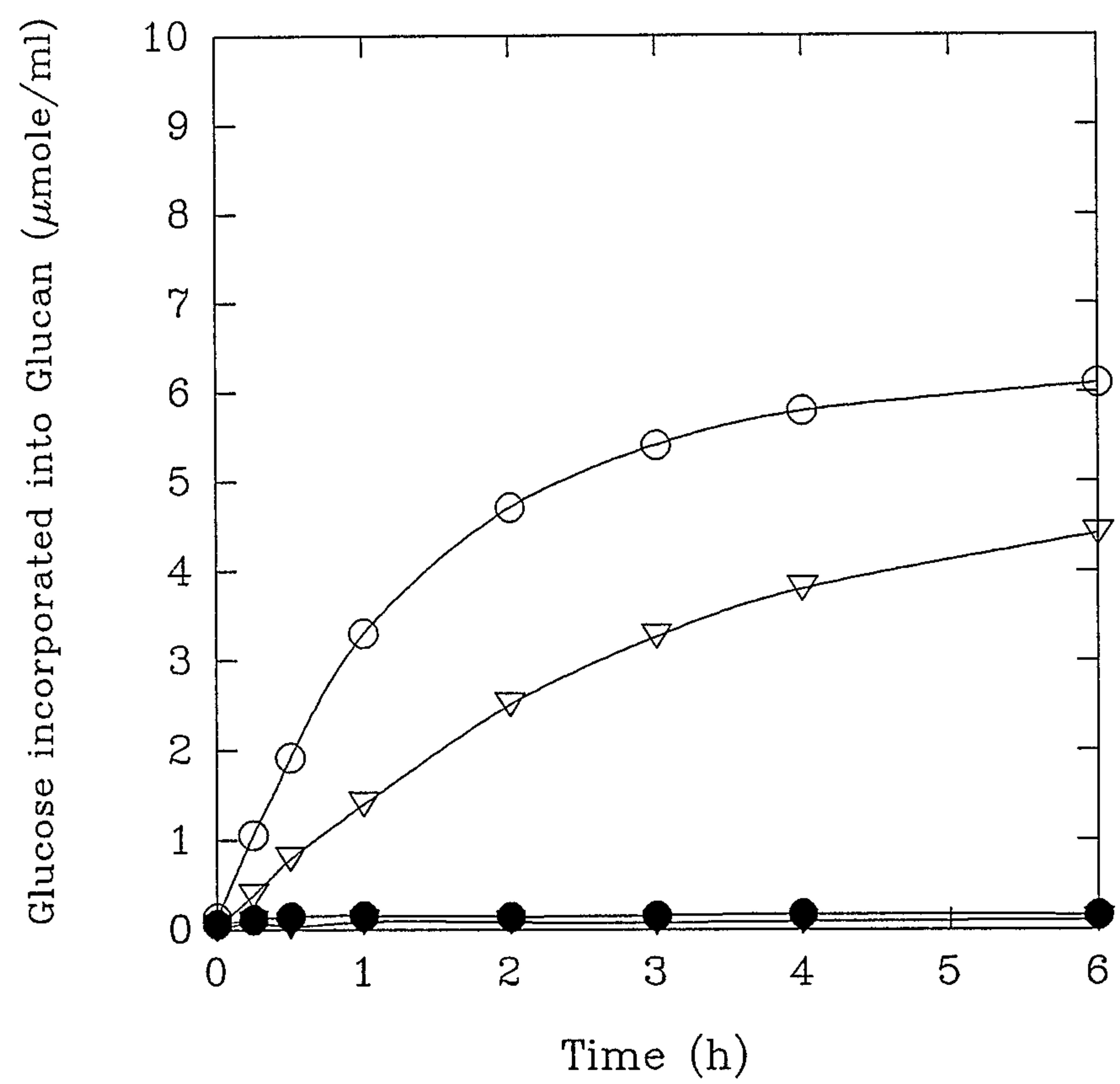
All four GTFs reacted differently to the inclusion of primer dextran. GtfJ was similar to that of previously reported primer-dependent GTF-I activities, that are unable to produce glucan without exogenous primer (Fukui *et al.*, 1982; Takehara *et al.*, 1984; Fig 8.1A). Previous results had indicated that a glucan is produced by this enzyme in the presence of sucrose without exogenous primer but over a much longer time frame (up to 5 days; see previous chapter).

GtfK appears to be similar to the GTFP3 of *S. sobrinus* and the GtfD of *S. mutans* (Hanada and Takehara, 1991; Hanada and Kuramitsu, 1989). Although these activities have been classified as primer-dependent, they can still produce some glucan without primer (Fig 8.1B). As glucan production by GtfK in the absence of dextran T-10 was essentially linear (Section 7) there was no evidence that the glucan produced by GtfK stimulated its own activity.

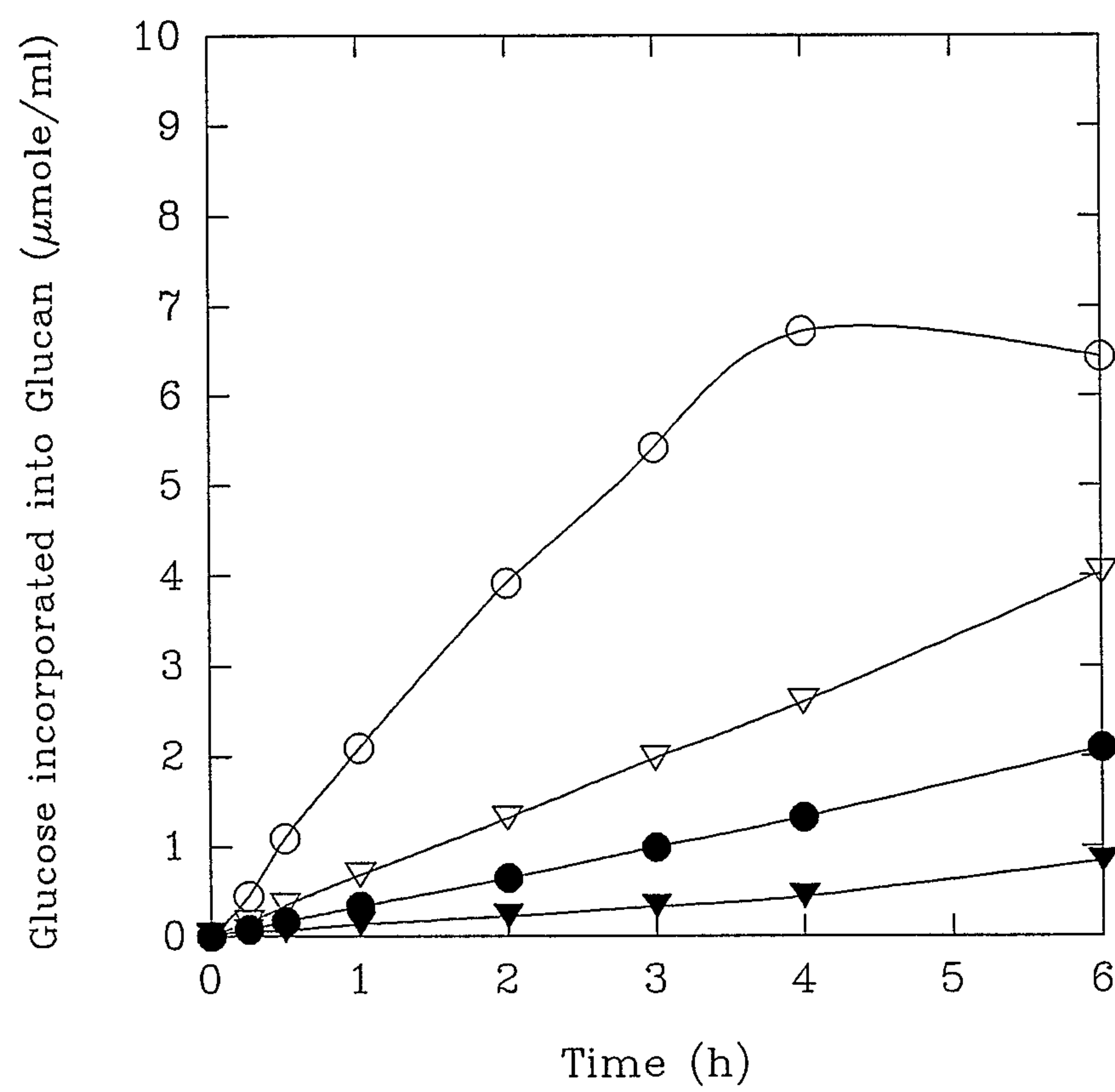
The GtfL activity was unique as the addition of 0.02% (w/v) dextran T-10 inhibited activity, as apparently did the formation of its own glucan (Fig 8.1C). As a consequence of this inhibition, little glucan was produced by the enzyme, with the concentration of enzyme having little effect on the final glucan production. Studies on a similar activity isolated from a human isolate of *S. salivarius* indicated that glucan synthesis was inhibited by dextran T-10 concentrations greater than 1µg/ml and also by the presence of a GTF-S (Sato and Inoue, 1991). Previous studies on the effect of Tween 80 on the primer-requirement of extracellular GTFs of *S. salivarius* indicated that dextran T-10 did not stimulate insoluble glucan production, but rather that it inhibited production at concentrations above 50µg/ml irrespective of whether or not Tween 80 was present in the growth medium (Wittenberger *et al.*, 1978). This result differed from that obtained with the GTFs of *S. sobrinus*. In this instance GTF activity was

Fig. 8.1.A-D. Glucan production by recombinant Gtfs, GtfJ (A), GtfK (B), GtfL (C) and GtfM (D) in the presence (hollow symbols) or absence (filled symbols) of 0.02% (w/v) dextran T-10 at 1x (▽, ▼) and 3x (○, ●) enzyme concentration.

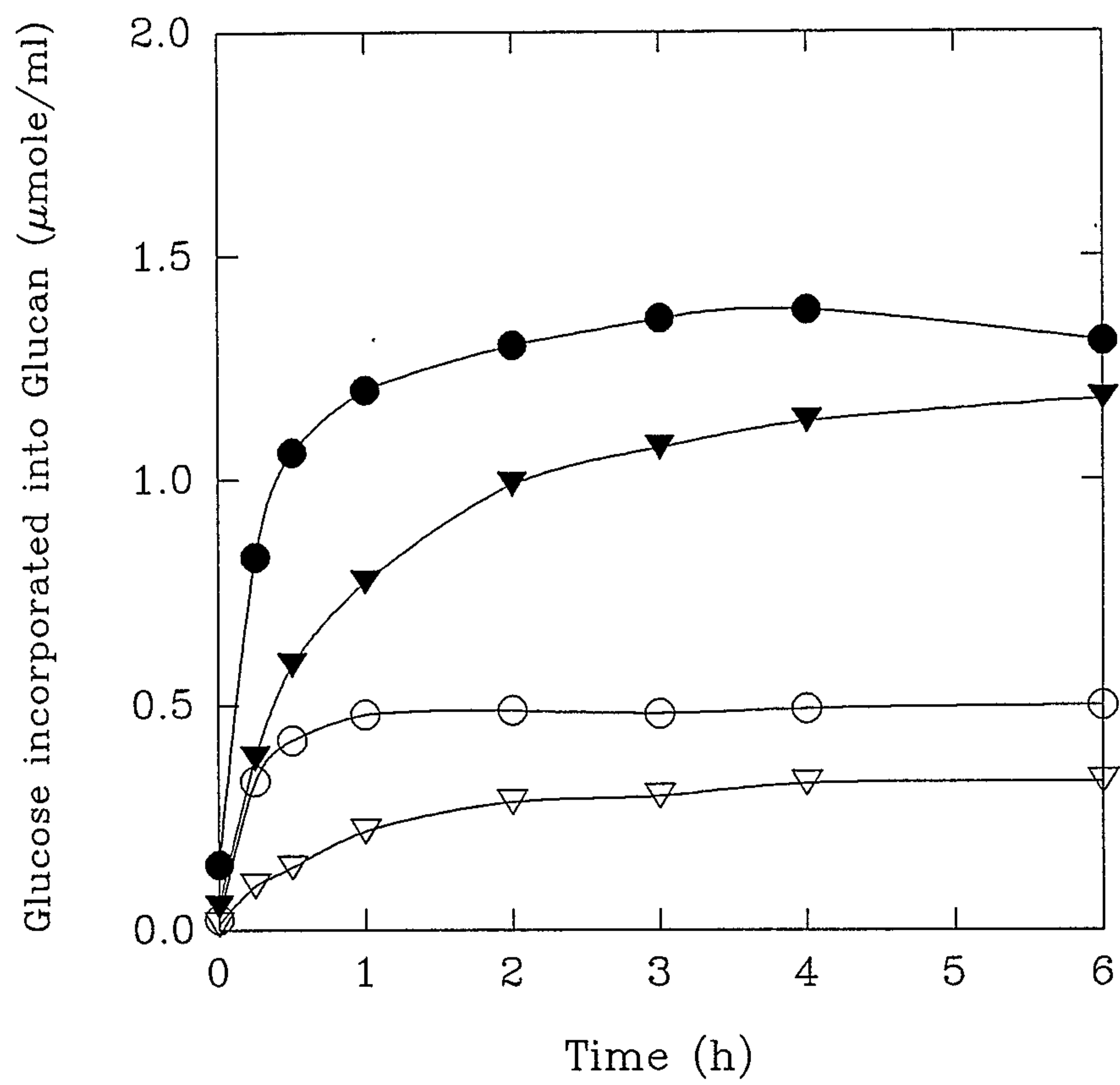
A. GtfJ



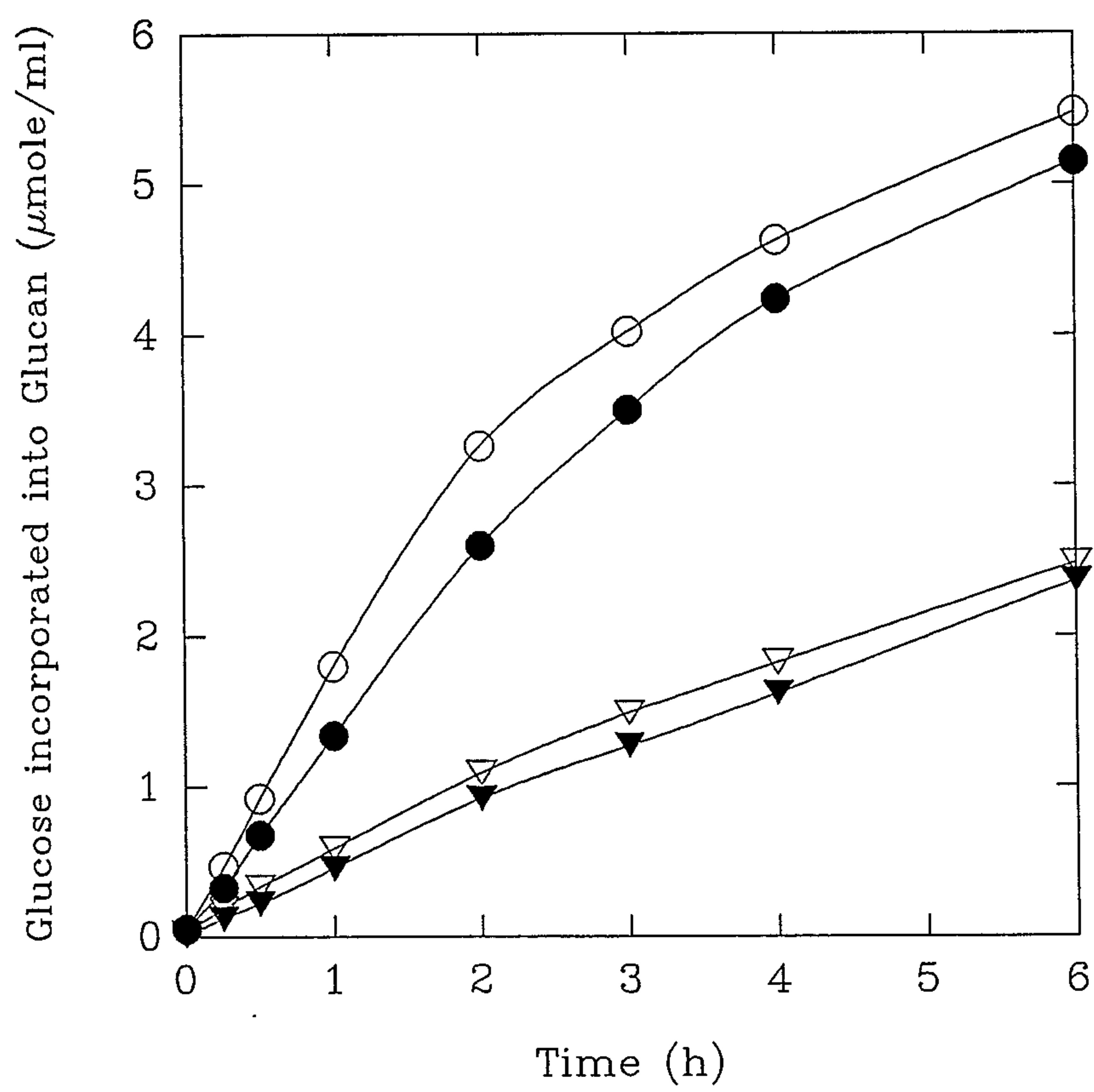
B. GtfK



C. GtfL



D. GtfM



stimulated by dextran T-10 after growth in medium lacking Tween 80, but not after growth in its presence. In fact in the latter case GTF activity was inhibited at dextran T-10 concentrations greater than 50 μ g/ml (Wittenberger *et al.*, 1978). Recent findings may explain this phenomenon as growth of *S. sobrinus* in Tween 80 appears to promote the secretion of two otherwise undetected primer-independent GTF-S activities and also large amounts of GTF-I (Walker *et al.*, 1990). No individual activities, however, have been reported from other oral streptococcal species which are inhibited by relatively low amounts of exogenous dextran as is the GtfL of *S. salivarius*.

Glucan production by GtfM was not stimulated or inhibited by dextran T-10 (Fig. 8.1D). Similar results have been found for the two primer-independent activities of *S. sobrinus* which also produce water-soluble glucans (McCabe, 1985; Yamashita *et al.*, 1988). This would suggest that the GTF acceptor reaction is not occurring and that all glucan production is proceeding via the autopolymerization pathway (Section 1.2). The primer-independent GTF-S of *S. sobrinus* that produces a high molecular weight glucan binds to glucan, but appears to lack a catalytic site for the polymerization of exogenous glucan (McCabe, 1985).

8.3.3 Effect of Dextran Concentration on Glucan Production

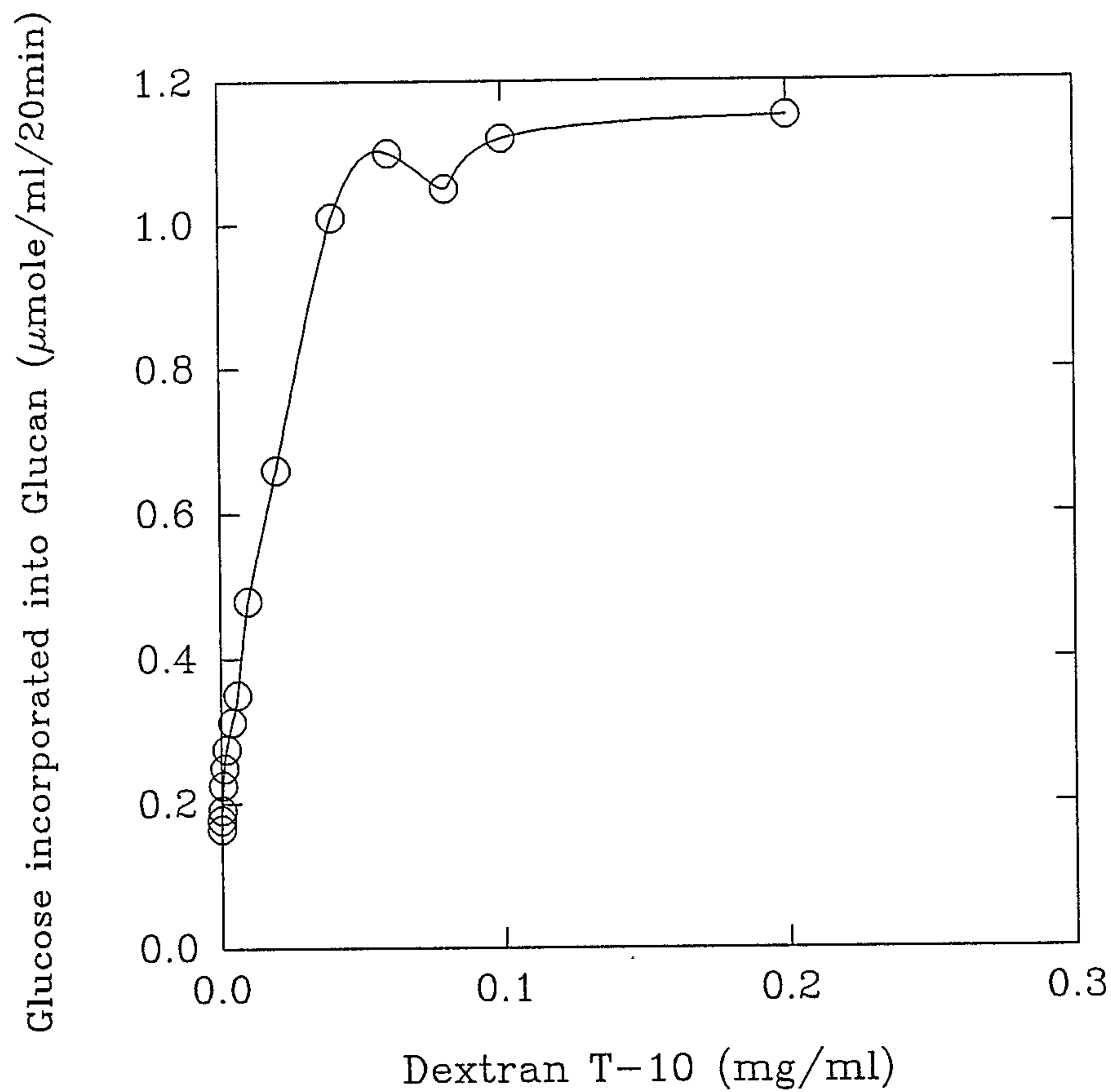
The effect of dextran concentration on glucan production was determined under standard assay conditions at pH 6.0. Both the GtfJ and GtfK enzymes were stimulated by increasing concentrations of dextran T-10 (Figs. 8.2A.I and 8.2B.I). However, Lineweaver Burk plots were not linear (Figs. 8.2A.II and 8.2B.II). The steep downwards inflection at high dextran concentrations has previously been observed by Mayer *et al.* (1981) who reported that this was the result of enzyme activation by dextran T-10 at these concentrations.

GtfL was strongly inhibited by exogenous dextran T-10, with maximum inhibition occurring at concentrations greater than 0.1mg/ml (Fig. 8.2C).

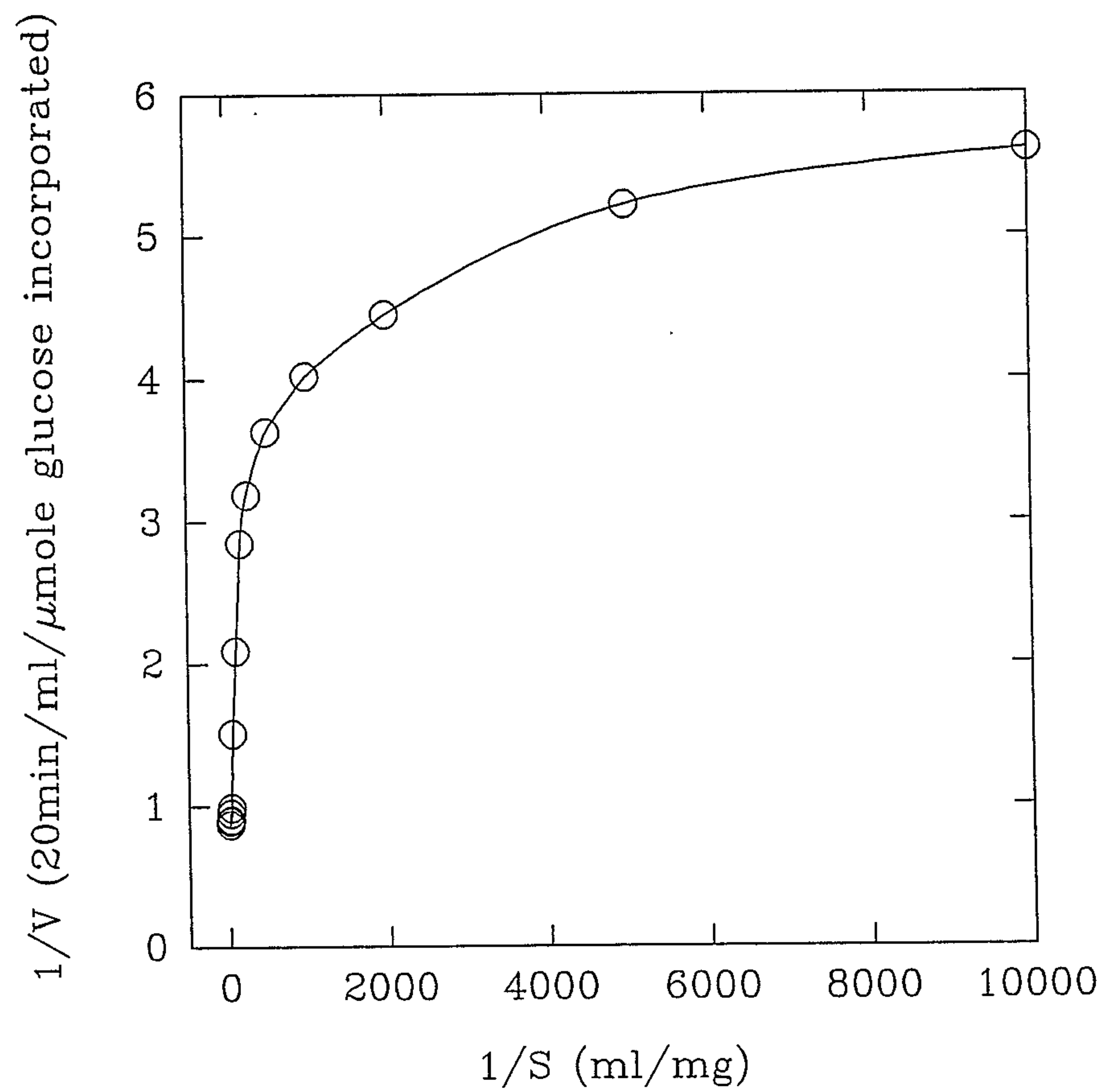
GtfM showed a slight increase in glucan production with increasing dextran concentrations (Fig 8.2D). The enzyme concentration of GtfM used in these experiments was 10 times that of the time course experiments (Fig. 8.1D), where there was apparently no significant stimulation with dextran T-10. A time course

Fig. 8.2. Effect on dextran T-10 concentration on glucan synthesis by recombinant GTF activities, GtfJ (A.I), GtfK (B.I), GtfL(C) and GtfM (D). Lineweaver-Burk plots were constructed for GtfJ (A.II) and GtfK (B.II).

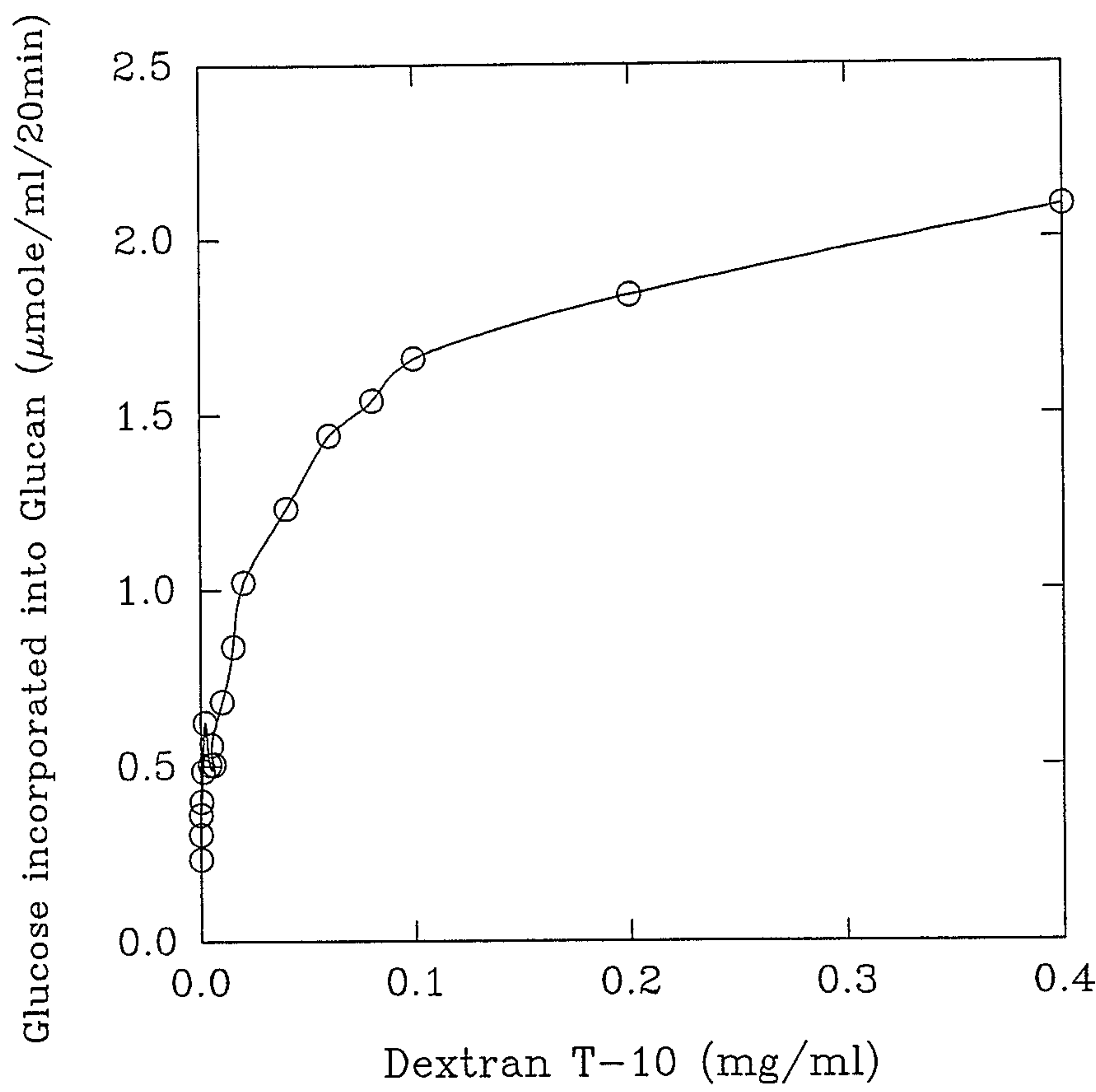
A.I GtfJ



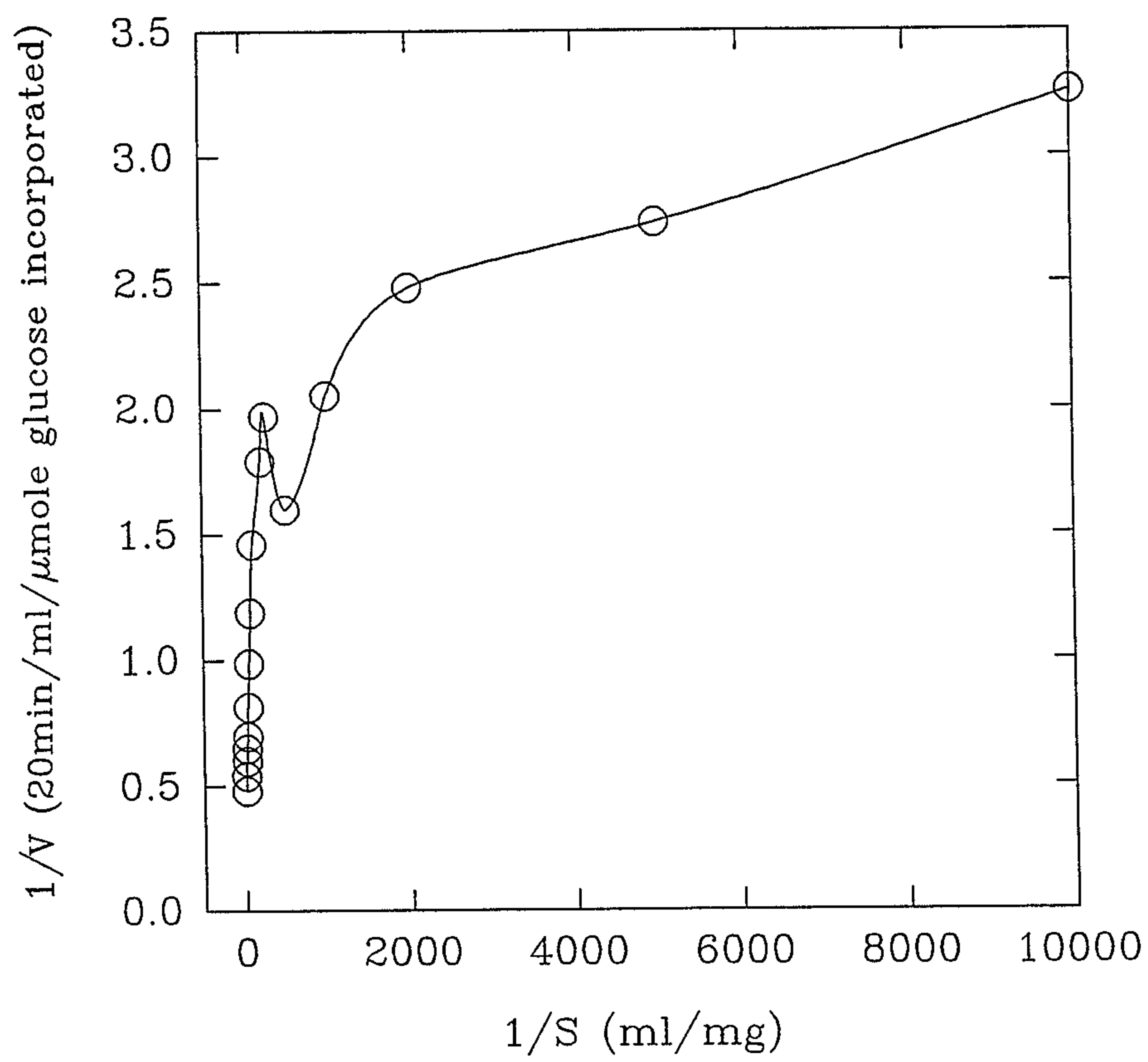
A.II



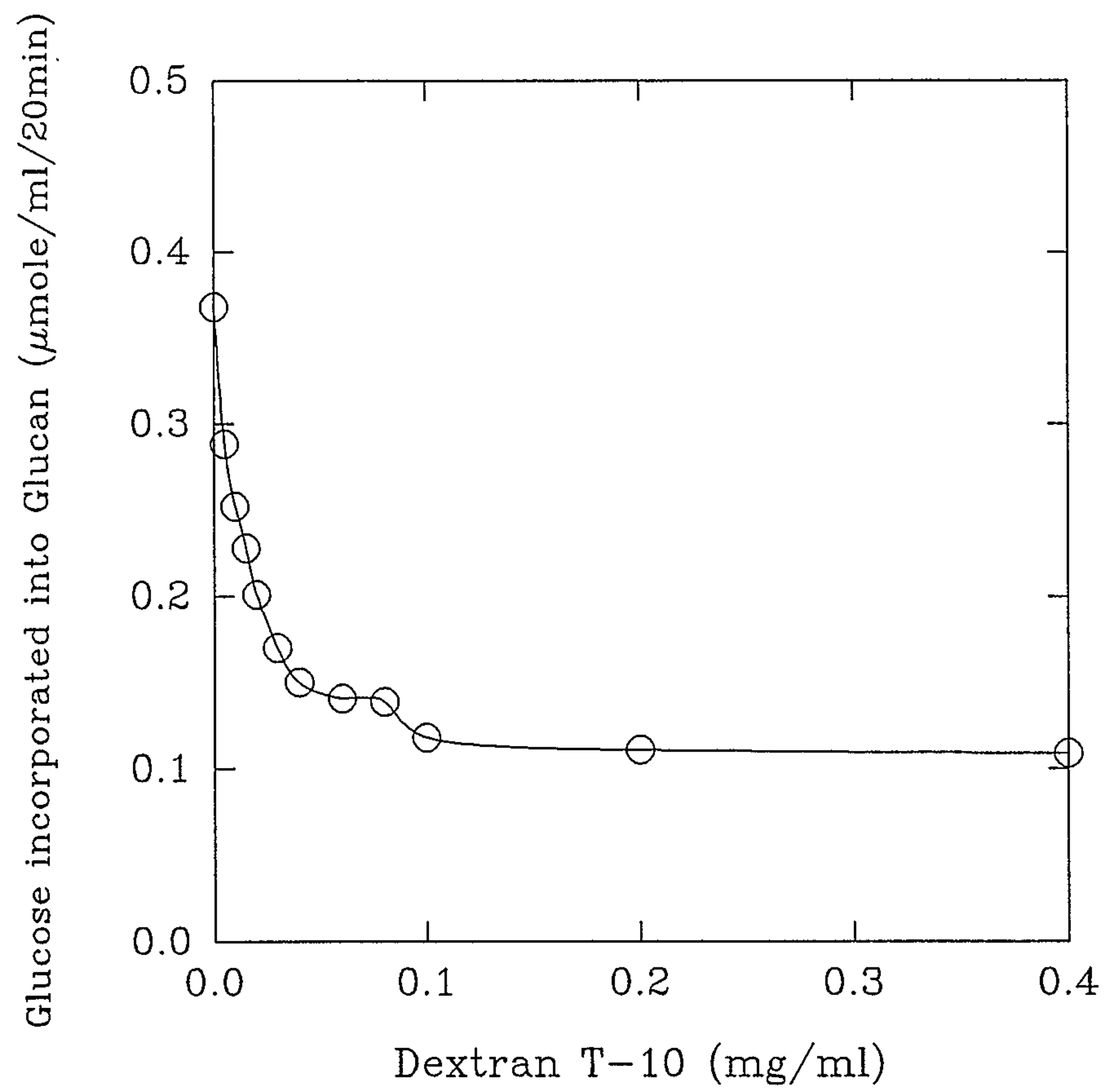
B.I GtfK



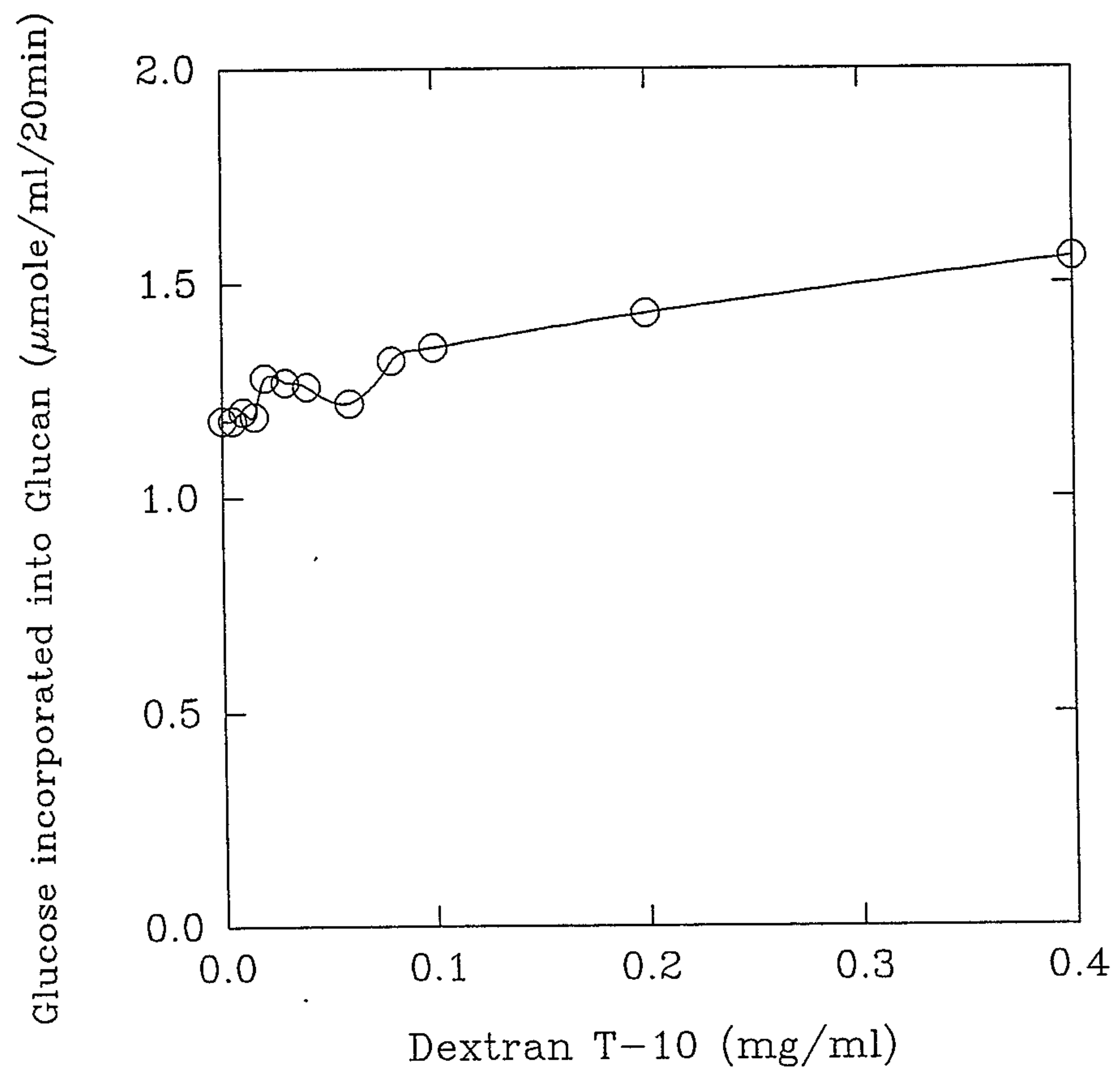
B.II



C. GtfL



D. GtfM



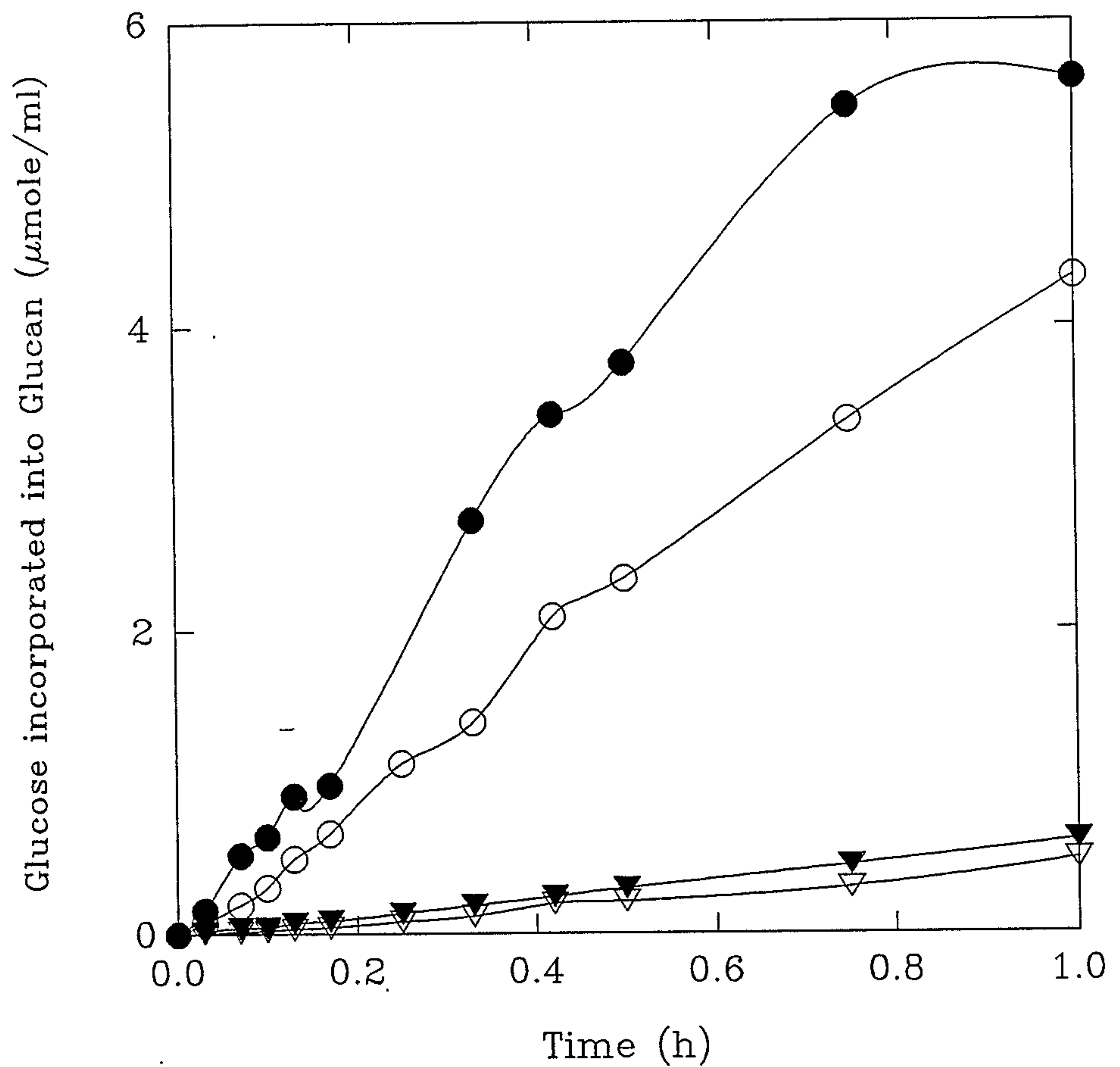


Fig. 8.3. Glucan production by GtfM with (filled symbols) and without (hollow symbols) dextran T-10 at the enzyme concentration used for K_m determinations (●, ○) (Fig. 8.2D), and the concentration used for time course experiments (▼, ▽) (Fig. 8.1D).

experiment was therefore carried out using this much higher concentration of enzyme and comparison made with the results using the lower concentration (Fig. 8.3). The result confirmed a slight enhancement of glucan synthesis by GtfM in the presence of dextran T-10 at the higher enzyme concentration. This increase may have been due either to a slight stimulation of GtfM by dextran T-10 under these conditions or to an increased entrapment of radioactively labelled sucrose by the greater amounts of dextran synthesized under these conditions (in other words an artifact of the assay method).

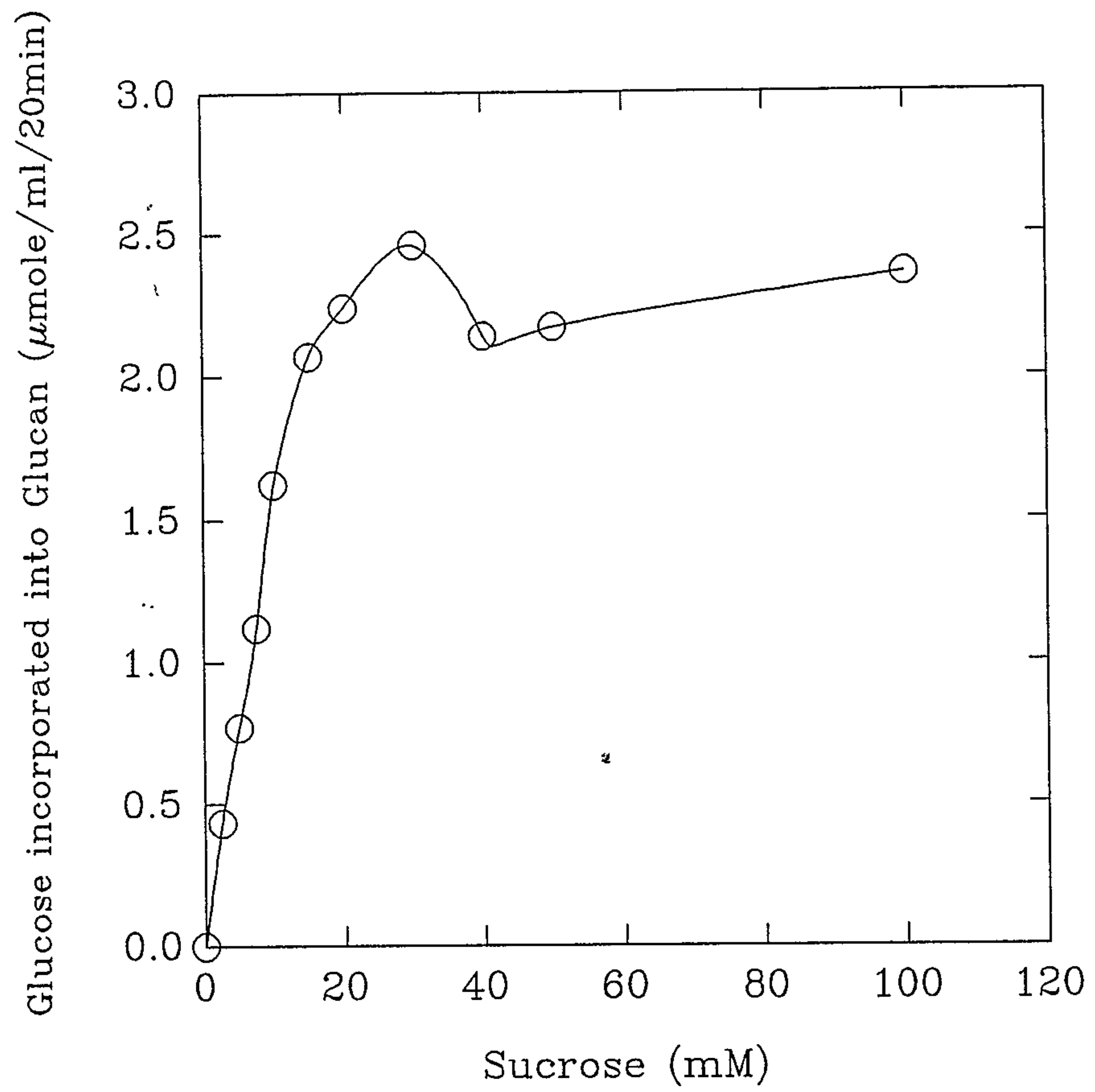
The concentration of dextran T-10 used routinely in assays for GTF activities (0.2mg/ml) maximally inhibited GtfL while maximally stimulating GtfJ and GtfK.

8.3.4 Effect of Sucrose Concentration on Glucan Production

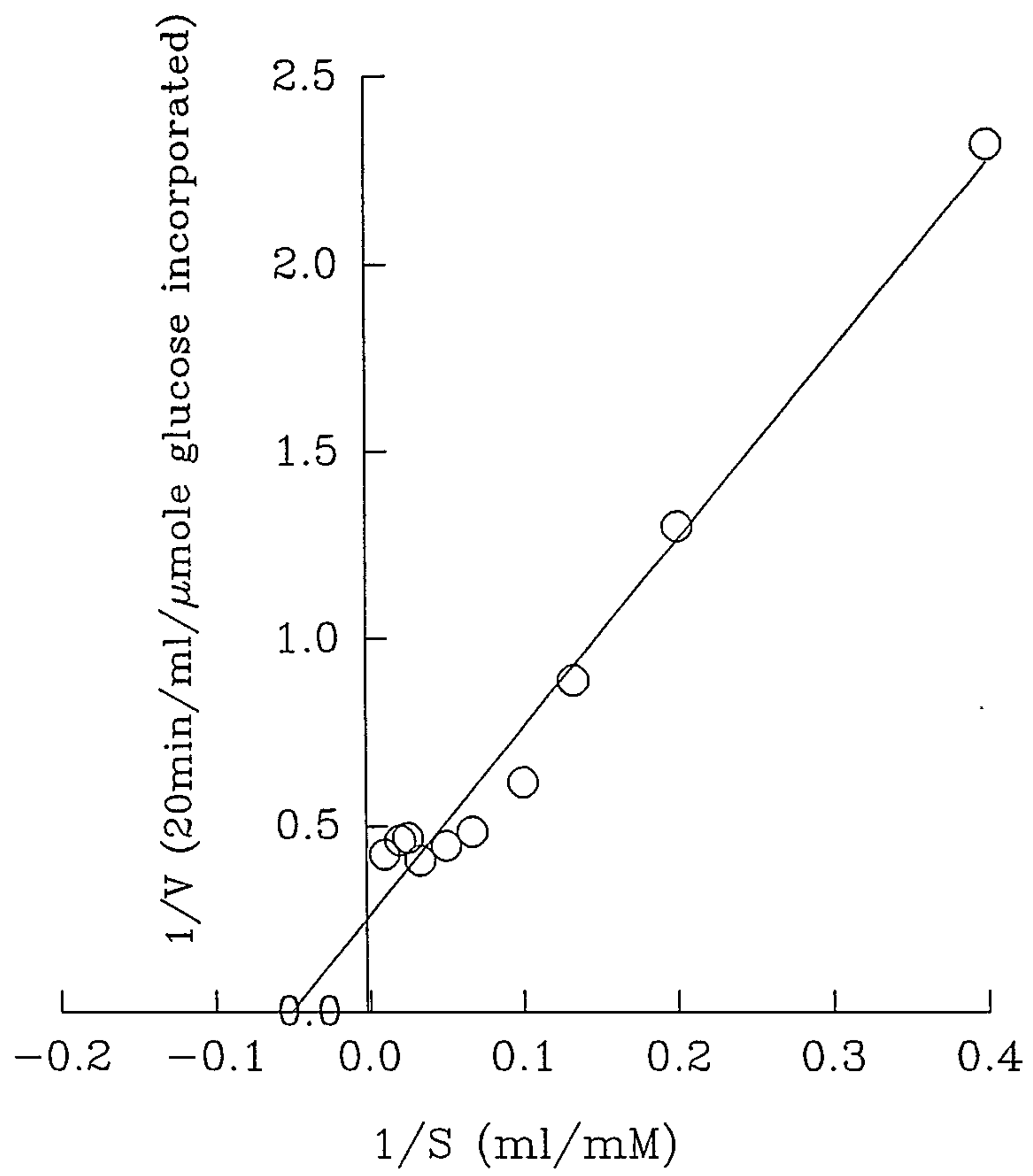
The K_m for sucrose, with and without the addition of dextran T-10, was calculated for each GTF expressed in *E. coli* except for GtfJ which did not produce glucan without primer. V_{max} values were not calculated as the amount of enzyme used in the experiments was arbitrary and therefore not known. K_m values were originally calculated based on the 'initial' rate of enzyme activity at a given concentration of sucrose. This 'initial' rate of enzyme activity was the amount of glucan formed in the first 20min (8.2.4). However, product inhibition was noted for GtfL at the higher sucrose concentrations used, so the 'initial' rate for this enzyme was based on the amount of glucan formed in the first 10min for each concentration of sucrose. The K_m values for sucrose for each of the four GTFs of *S. salivarius* were calculated from the data obtained (Fig. 8.4) and were within the range of 7 to 20mM (Table 8.5). In contrast, the primer-independent GTFs of *S. sobrinus* have K_m values for sucrose of 3.9mM and 145mM (Hanada *et al.*, 1987; Yamashita *et al.*, 1988). The addition of dextran T-10 had little effect on K_m , but increased the V_{max} for GtfJ, GtfK and GtfM as expected. Although the increase in V_{max} for GtfM was not as dramatic as for GtfJ and GtfK, this activity was stimulated by the presence of dextran T-10. Dextran T-10 reduced the V_{max} of GtfL, but had little effect on the K_m value for sucrose suggesting that dextran T-10 acts as a non-competitive inhibitor of glucan production by GtfL (Table 8.5).

Fig. 8.4. Effect of sucrose concentration (I) on glucan-synthesis by recombinant GTF activities, GtfJ (A), GtfK (B), GtfL(C) and GtfM (D), in presence (O) and absence (●) of 0.02% (w/v) dextran T-10. Lineweaver-Burk plots were also constructed (II).

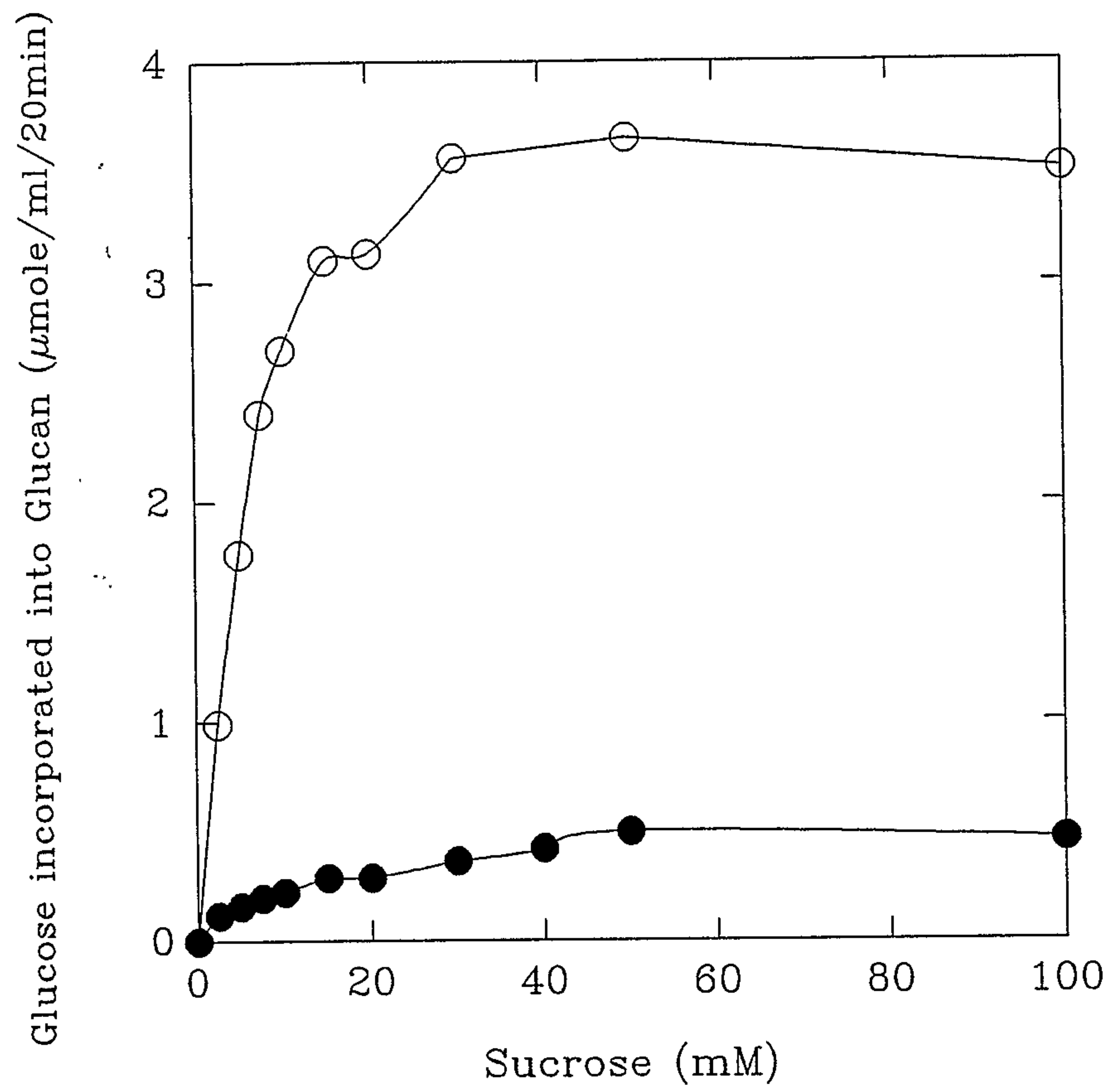
I. A GtfJ



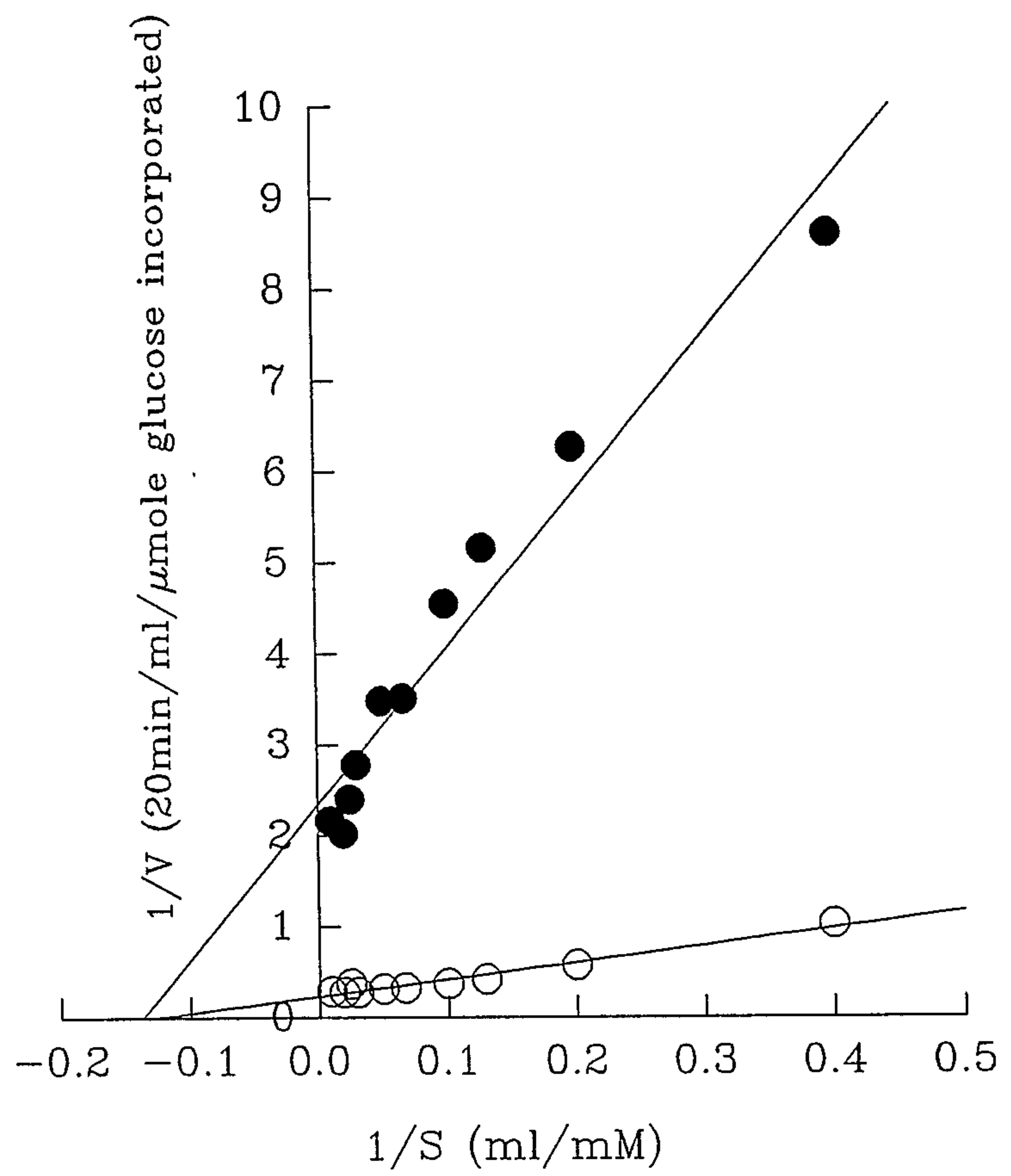
II.A



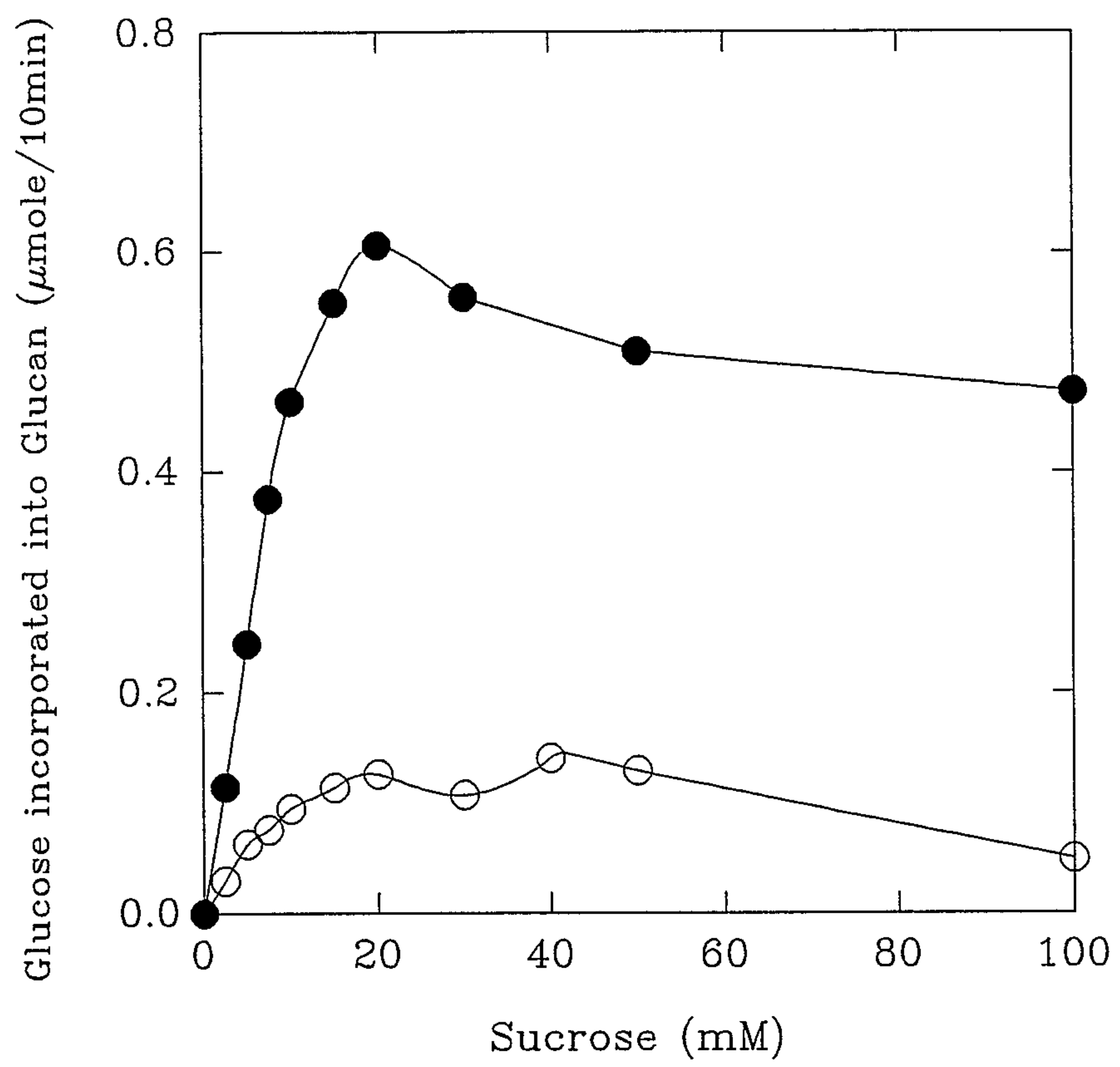
I.B GtfK



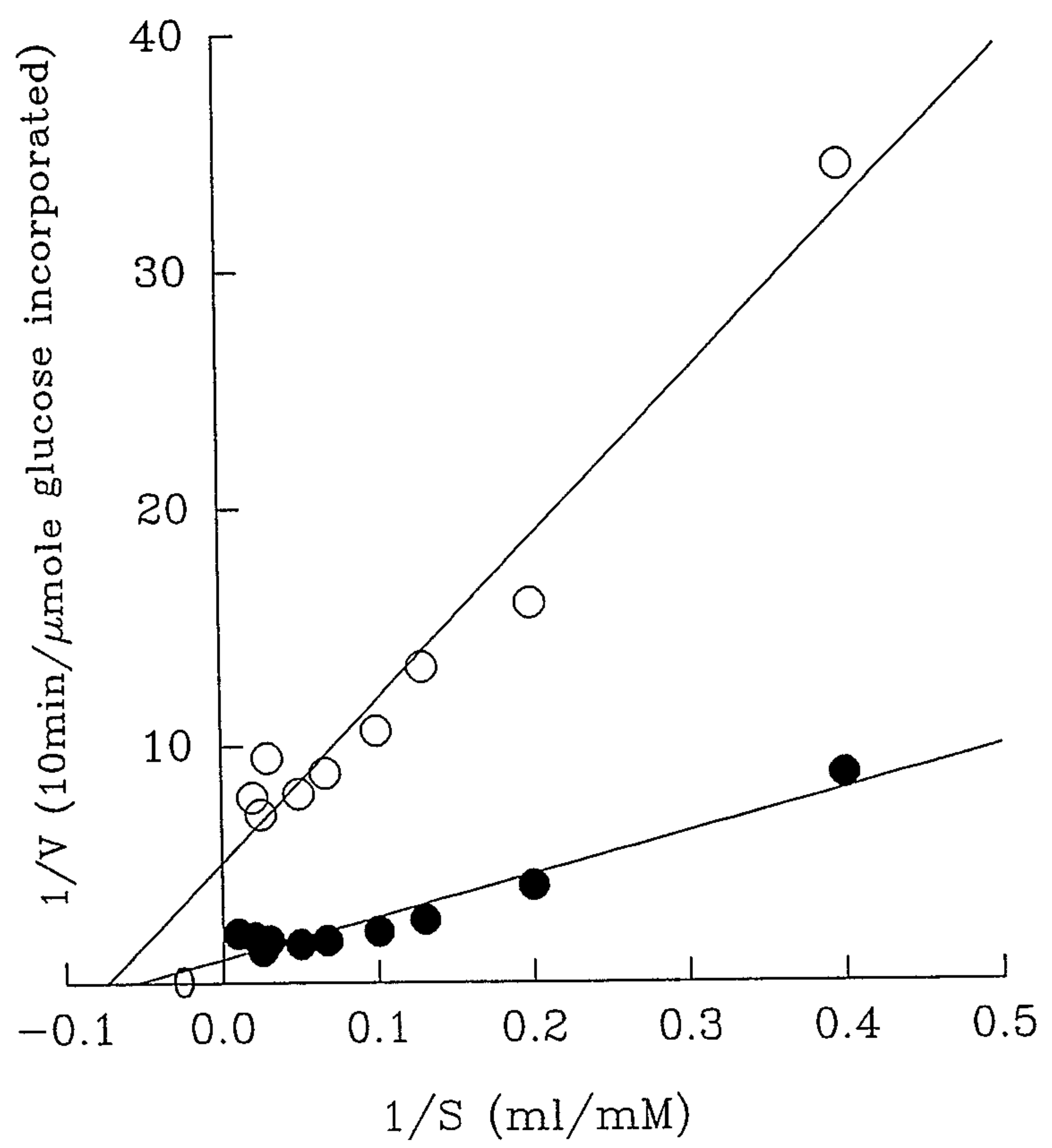
II.B



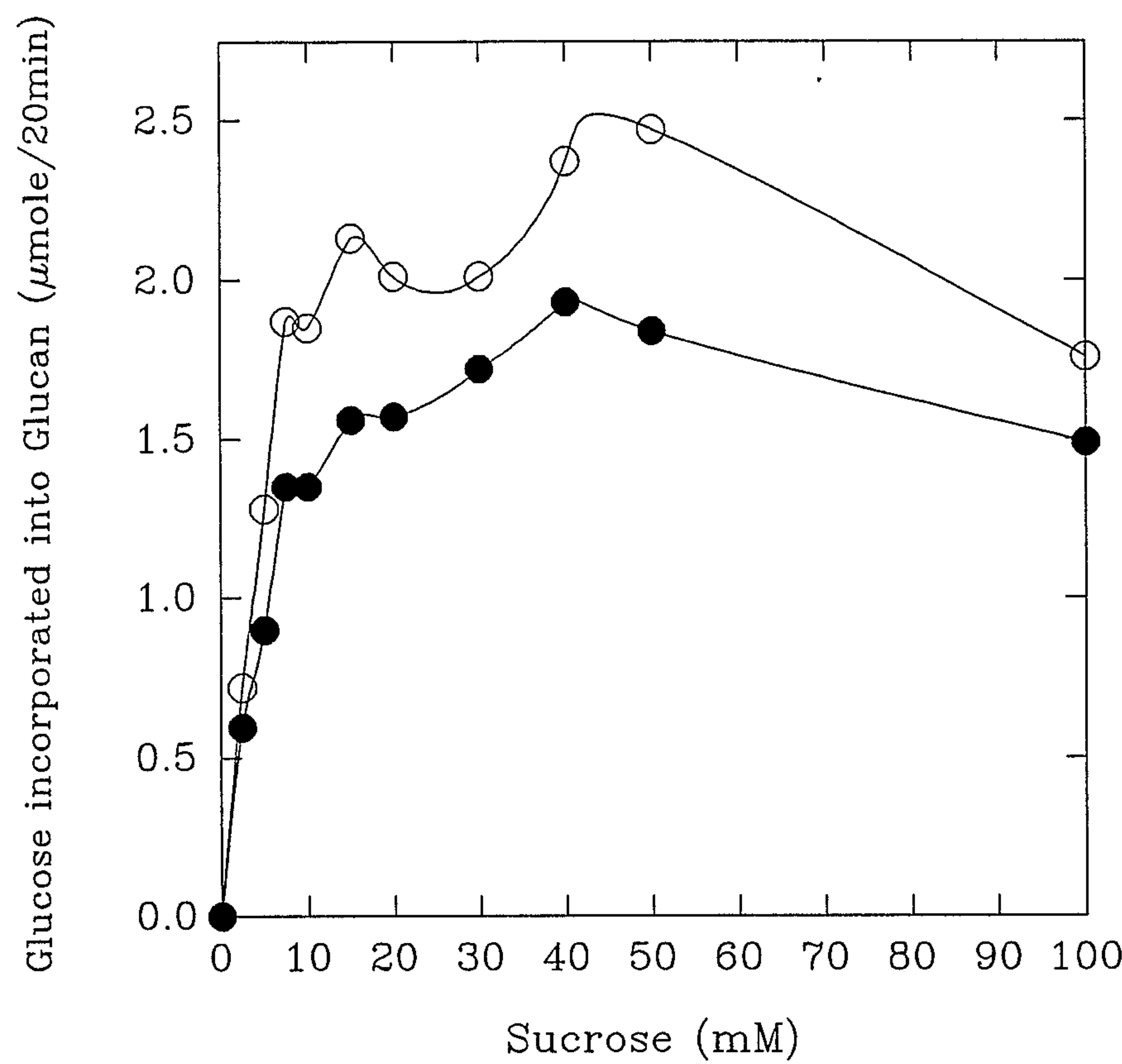
I.C Gtfl



II.C



I.D GtfM



D.II

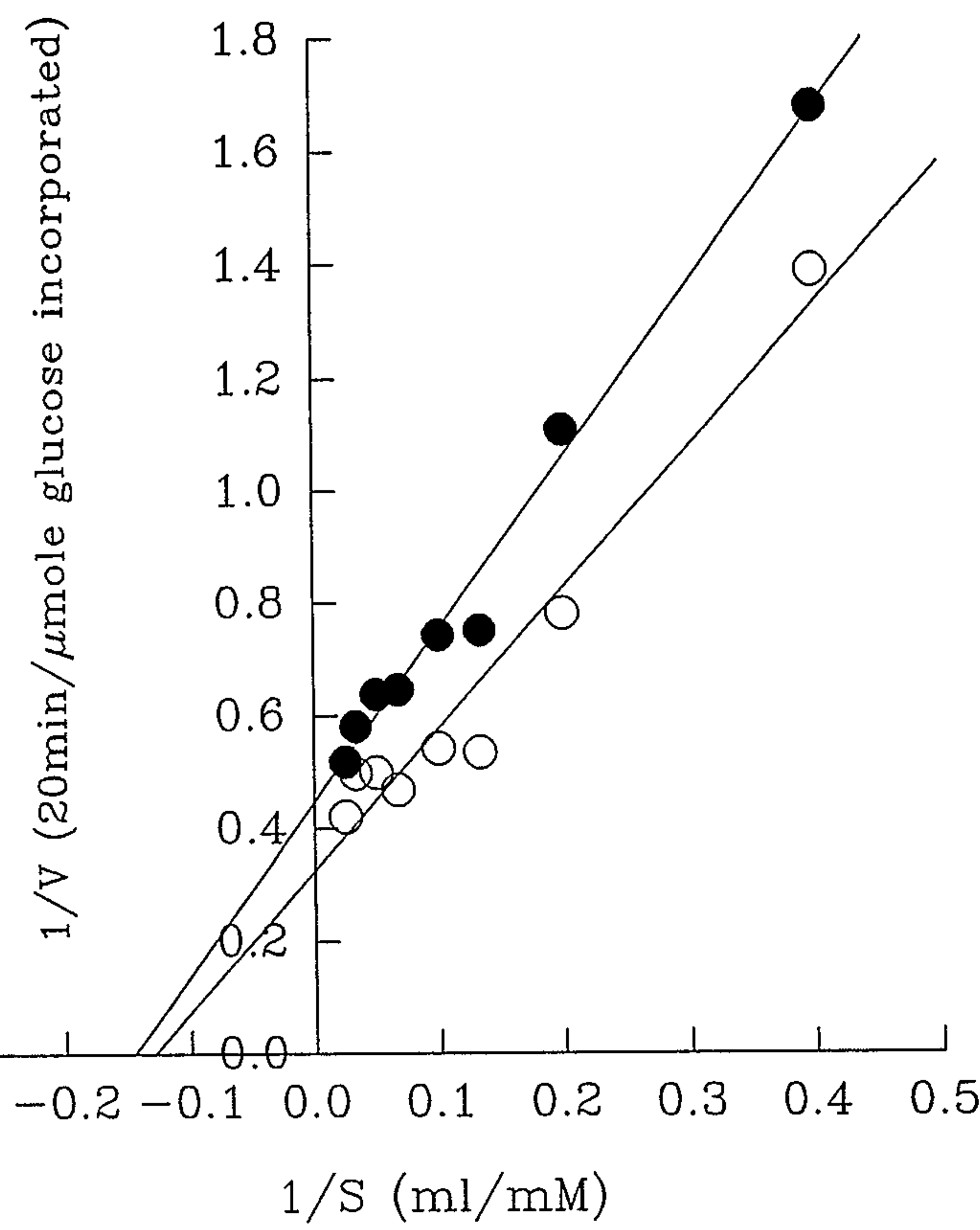


Table 8.5. K_m for sucrose in the presence and absence of 0.02% (w/v) dextran T-10

GTF	K_m Sucrose (mM)	
	- Dextran T-10	+ Dextran T-10
GtfJ	-	20
GtfK	7	8
GtfL	17	14
GtfM	7	7

Table 8.6. Effect of pH on glucan synthesis by recombinant Gtf activities.

pH	Glucan Production (μ mole glucose incorporated/ml)			
	GtfJ	GtfK	GtfL	GtfM
3.7	0.0	0.2	0.0	0.0
4.6	0.0	0.2	2.3	0.3
5.2	0.4	0.6	<u>2.5</u>	0.8
5.6	<u>0.5</u>	0.7	1.8	<u>1.3</u>
6.4	0.4	<u>0.7</u>	1.4	1.1
7.2	0.3	0.6	0.8	0.7
7.9	0.0	0.4	0.6	0.1
8.7	0.0	0.3	0.0	0.0
9.0	0.0	0.2	0.0	0.0

* Maximum glucan production is underlined and in bold.

8.3.5 Effect of pH on Glucan Production

The maximum activities of the four GTFs of *S. salivarius* expressed in *E. coli* occurred within the range pH 5.2 - pH 6.4 (Table 8.6). GtfM activity was maximal at pH 5.6 despite having a more alkaline pI than the other enzymes (Section 5; Section 6). The range of pH over which an enzyme was active differed. For example GtfJ was active over a limited pH range of 5.2-7.2, whereas GtfK produced glucan over the entire pH range tested.

As much higher activities were noted for GtfL at pHs lower than the standard assay pH of 6.0, the effect of pH on dextran inhibition was also tested. At the enzymes optimum pH for activity, dextran inhibition was no longer apparent (Table 8.7). The optimum pH for activity for this enzyme is very close to its pI value, as determined by isoelectric focusing (Chapter 5) and from the deduced amino acid sequence (Chapter 6). At this pH the enzyme has no overall net charge and may aggregate, which may be responsible for this apparent change in enzyme activity. Aggregation of GTFs in high salt conditions, or in low concentrations of hydrogen peroxide, has previously been suggested to be responsible for the production of insoluble glucan by GTF-S enzymes (Mukasa *et al.*, 1979; McAlister *et al.*, 1989).

8.3.6 Acceptor Efficiency of Glucans Produced by Primer-Independent Activities

Approximately 0.04mg/ml of the glucans produced by the GtfK, GtfL and GtfM enzymes were used as primers for the GtfJ and GtfK activities (Table 8.8; Section 8.2.6). Product inhibition prevented higher concentrations of glucan being produced by GtfL.

The glucans produced by GtfK, GtfL and GtfM all acted as acceptors for glucan synthesis by GtfJ, with the glucan produced by GtfM the most efficient acceptor (Fig. 8.5A). In contrast to previous results, using dextran T-10 as primer, soluble intermediates were noted when the soluble glucan produced by GtfK was used as a primer for GtfJ (Fig. 8.5B). No soluble intermediates were produced by GtfJ in the presence of the glucan synthesized by GtfM or the glucan produced by GtfL (data not shown). In all cases the initial rates of glucan synthesis by GtfJ were high. However, glucan synthesis was virtually complete within 2h presumably

Table 8.7. Effect of pH on dextran inhibition of glucan synthesis by GtfL

pH	Glucan Production (μ mole glucose incorporated/ml)	
	- Dextran T-10	+ Dextran T-10 *
5.2	0.6	0.6
5.6	0.4	0.2
6.4	0.4	0.1

* Dextran T-10 concentration was 0.02% (w/v)

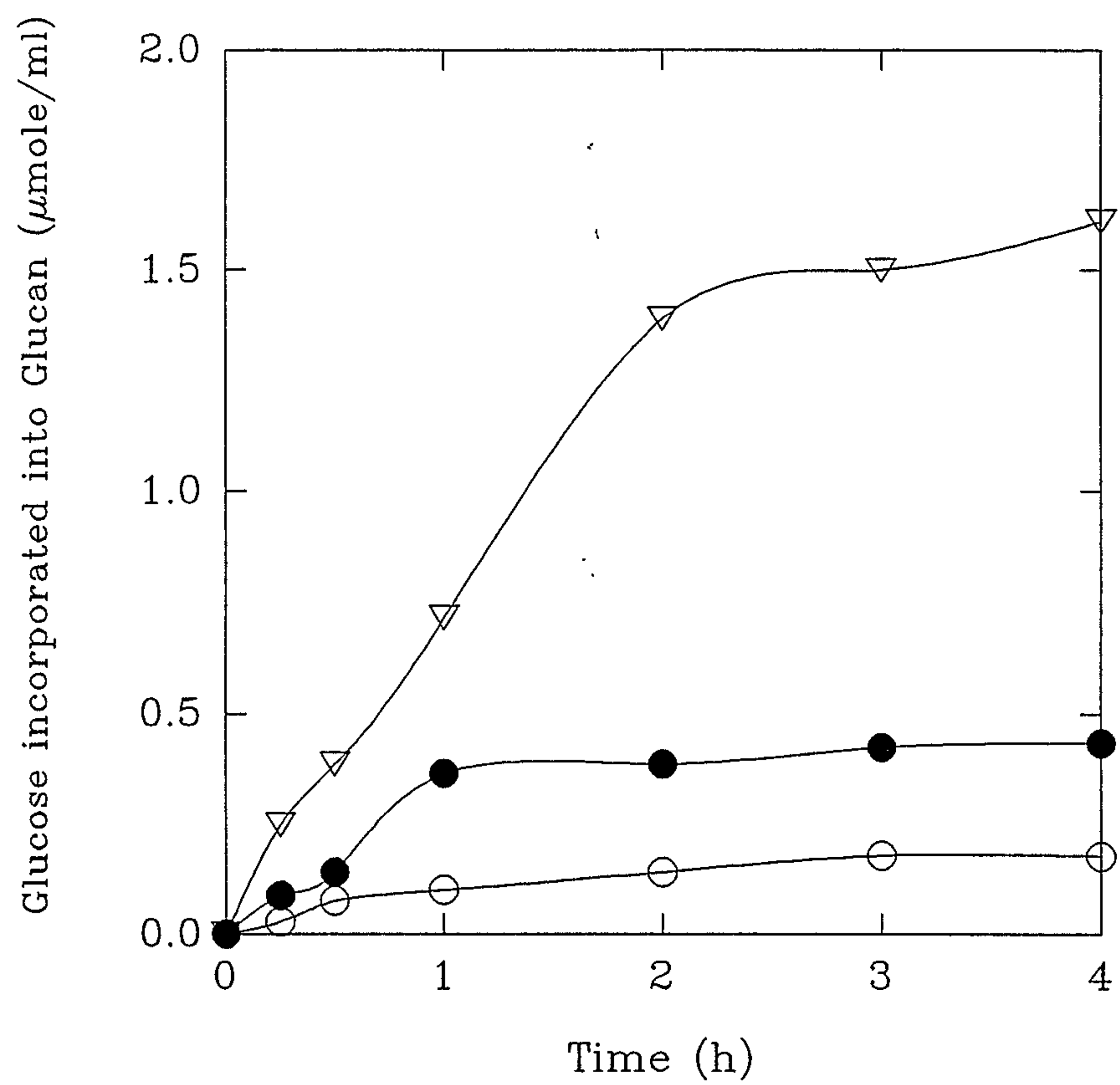
Table 8.8. Concentration of glucan used as acceptors for glucan synthesis by GtfJ and GtfK

Enzyme Producing Glucans	Amount of Glucan Formed (mg/ml)
GtfK	0.041
GtfL	0.049
GtfM	0.039

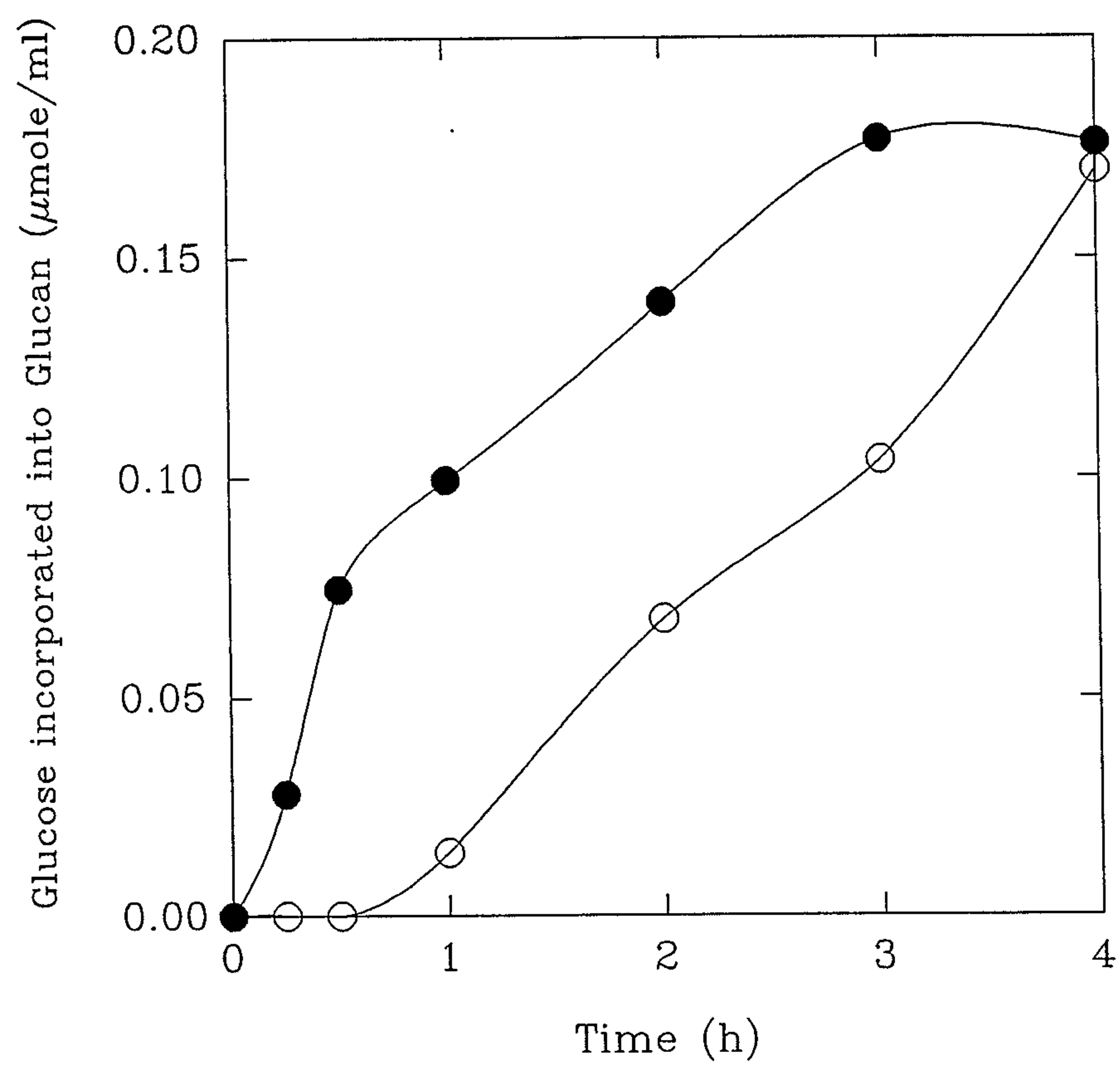
Fig. 8.5. A. Priming effectiveness of glucans produced by the recombinant activities, GtfK (○), GtfL (●) and GtfM (▽), on glucan synthesis by GtfJ.

B. The insoluble (○) and total glucan (●) produced by GtfJ in the presence of the glucan produced by GtfK.

A.



B.



as a result of all available primer becoming insoluble by the addition of α -1,3-linked residues.

Glucans synthesized by GtfM and GtfL enzymes were able to prime GtfK activity. However the glucan produced by GtfK did not (Fig. 8.6A). This result was consistent with the results of the time course experiments where the production of glucan by GtfK in the absence of dextran T-10 was linear (Fig. 8.1B). Only soluble glucan was produced by GtfK in the presence of glucan synthesized by GtfM (data not shown). In the reaction of GtfK with the glucan produced by GtfL, both soluble and insoluble glucan were detected (Fig. 8.6B). The amount of soluble glucan was greater than that expected through autopolymerization by GtfK (8.3.2; Fig. 8.1.B; Fig. 8.6B).

As the priming ability of glucan produced by GtfK was very low for both GtfJ and GtfK, and some precipitation of glucan was noted during enzyme inactivation (8.2.6), further experiments were carried out to confirm the ineffectiveness of this glucan as an acceptor. The glucan product prepared for ^{13}C -NMR analysis (Chapter 7) was added to 20ml of assay mix at a concentration of 0.04mg/ml together with 300 μ l sonicated *E. coli* expressing GtfJ or GtfK and incubated at 37°C. Production of glucan was monitored by 75% (v/v) ethanol precipitation of 1ml aliquots of assay mix over a period of 2-4h. Similar experiments were carried out replacing the glucan produced by GtfK with 0.04mg/ml of dextran T-10. Unfortunately new bacterial sonicates (and therefore different enzyme concentrations) had to be prepared for these experiments so direct comparisons could not be made with previous experiments.

The result from this experiment differed from that previously obtained with GtfJ, with the glucan produced by GtfK effectively priming glucan synthesis (Fig. 8.7). However, the glucan was still a poor acceptor for GtfK activity, although the rate of synthesis of glucan increased over time (Fig. 8.8).

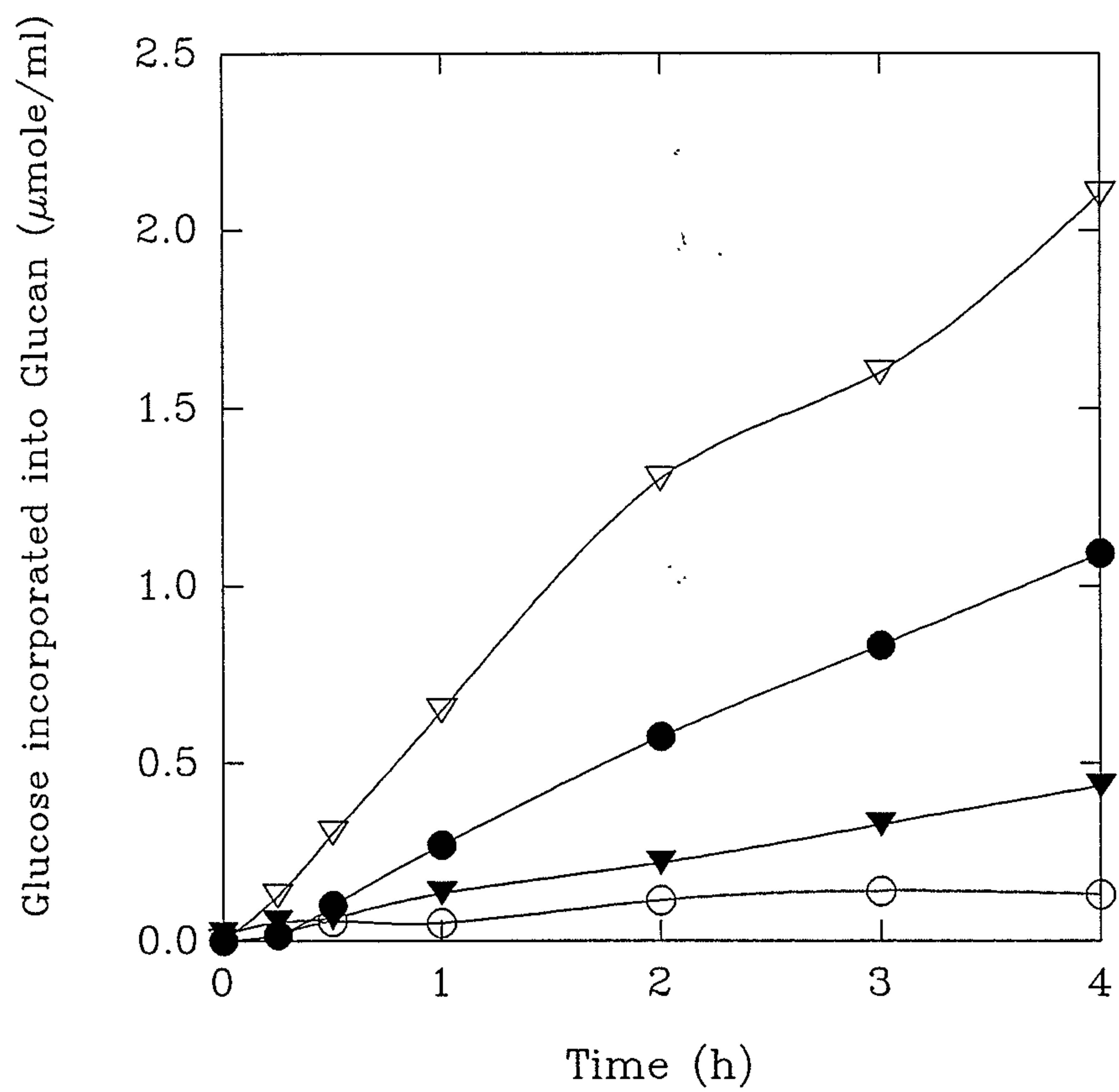
The ability of glucan to act as an acceptor for primer-dependent GTF-I activities has previously been demonstrated to depend upon its lengths of unbranched α -1,6-linked glucosyl residues (Walker and Schuerch, 1986). The glucans produced by GtfM and GtfK were both efficient acceptors for GtfJ activity. This is consistent with their structural analysis. Both the glucan produced

Fig. 8.6. A. Priming effectiveness of glucans produced by the recombinant activities, GtfK (○), GtfL (●) and GtfM (▽), on glucan synthesis by GtfK.

Production of glucan by GtfK in the absence of primer (▼) is also shown.

B. The insoluble (○) and total glucan (●) produced by GtfK in the presence of the glucan of GtfL.

A.



B.

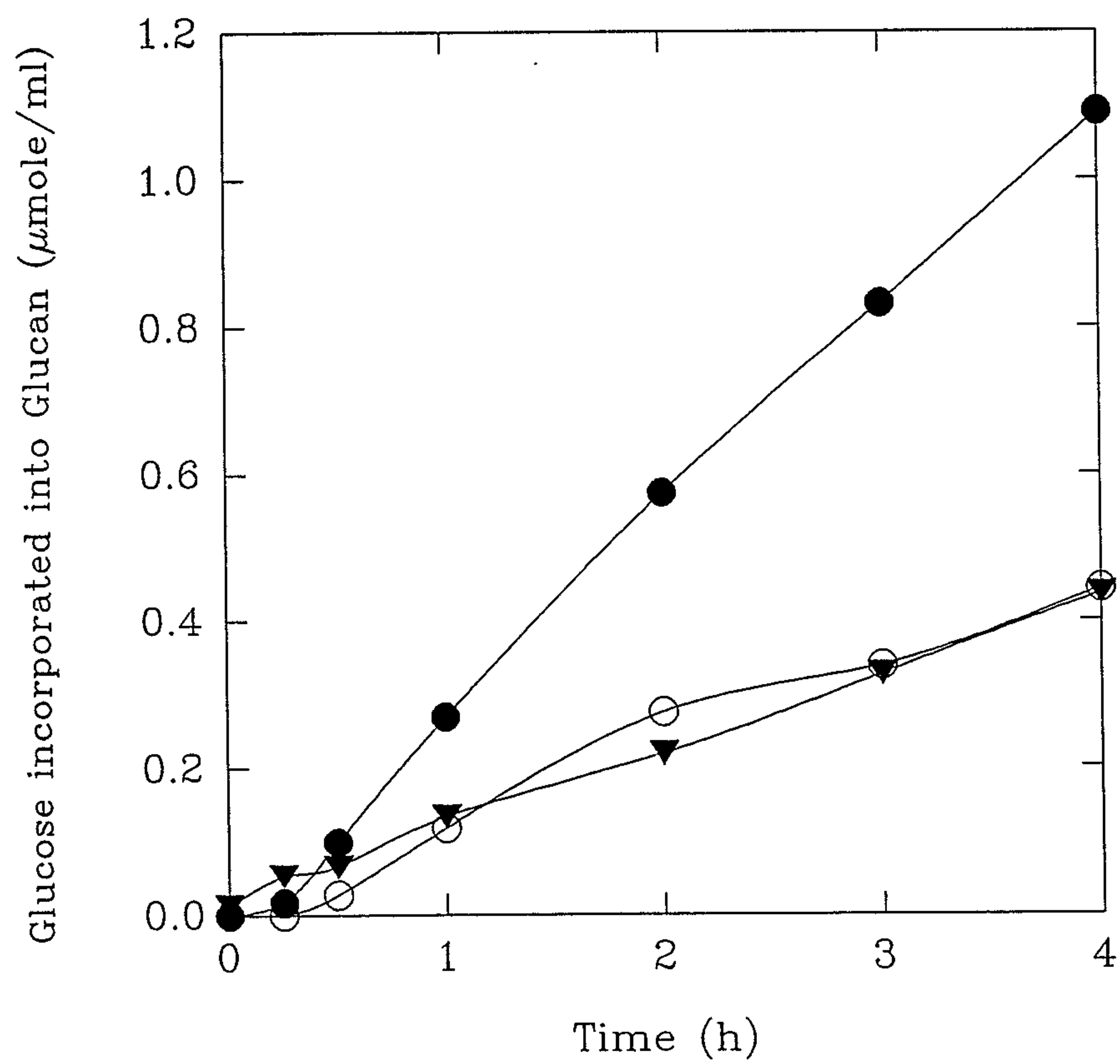


Fig. 8.7. Priming effectiveness of freeze-dried glucan produced by GtfK (○) and dextran T-10 (●) on glucan synthesis by GtfJ.

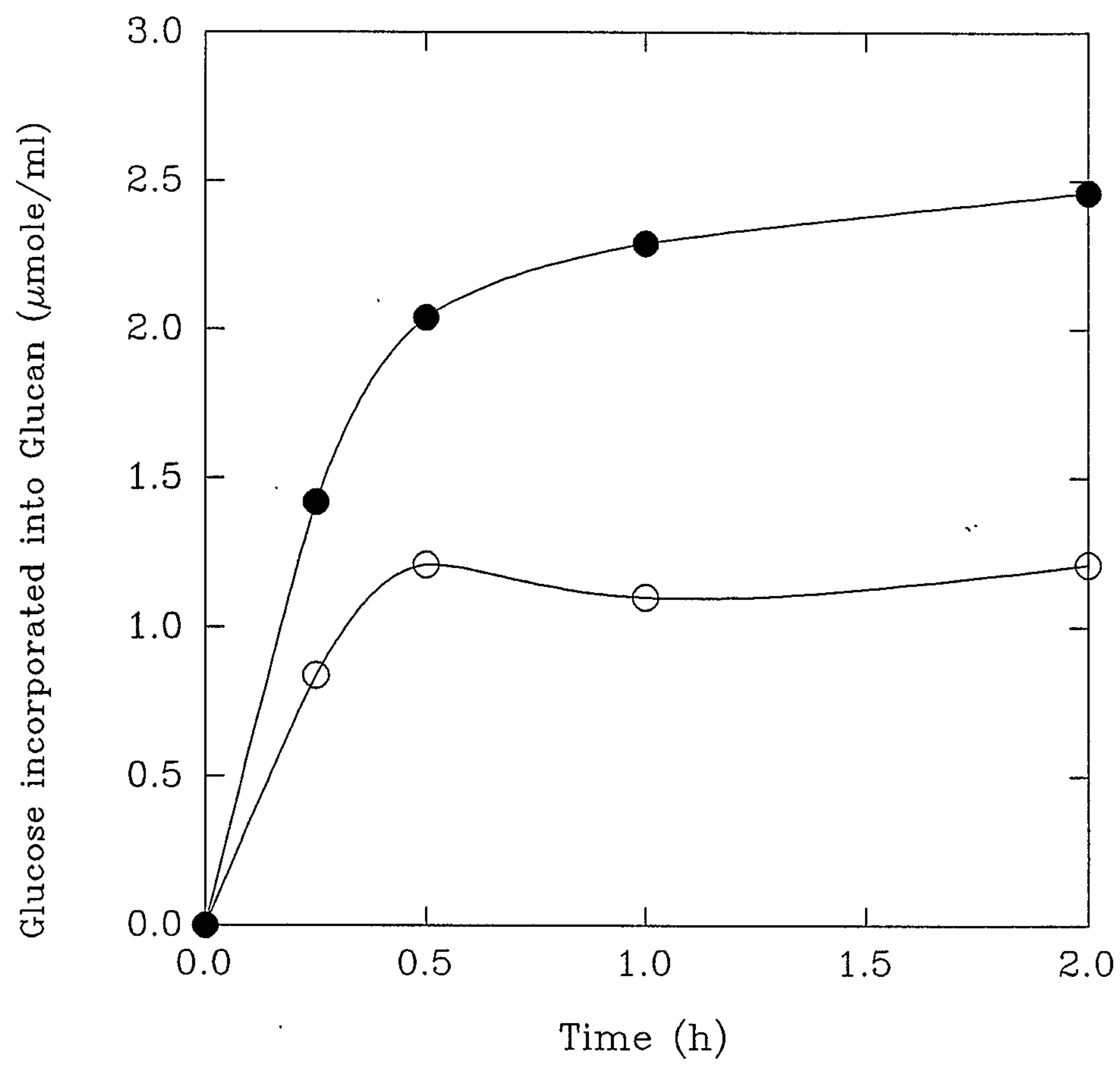
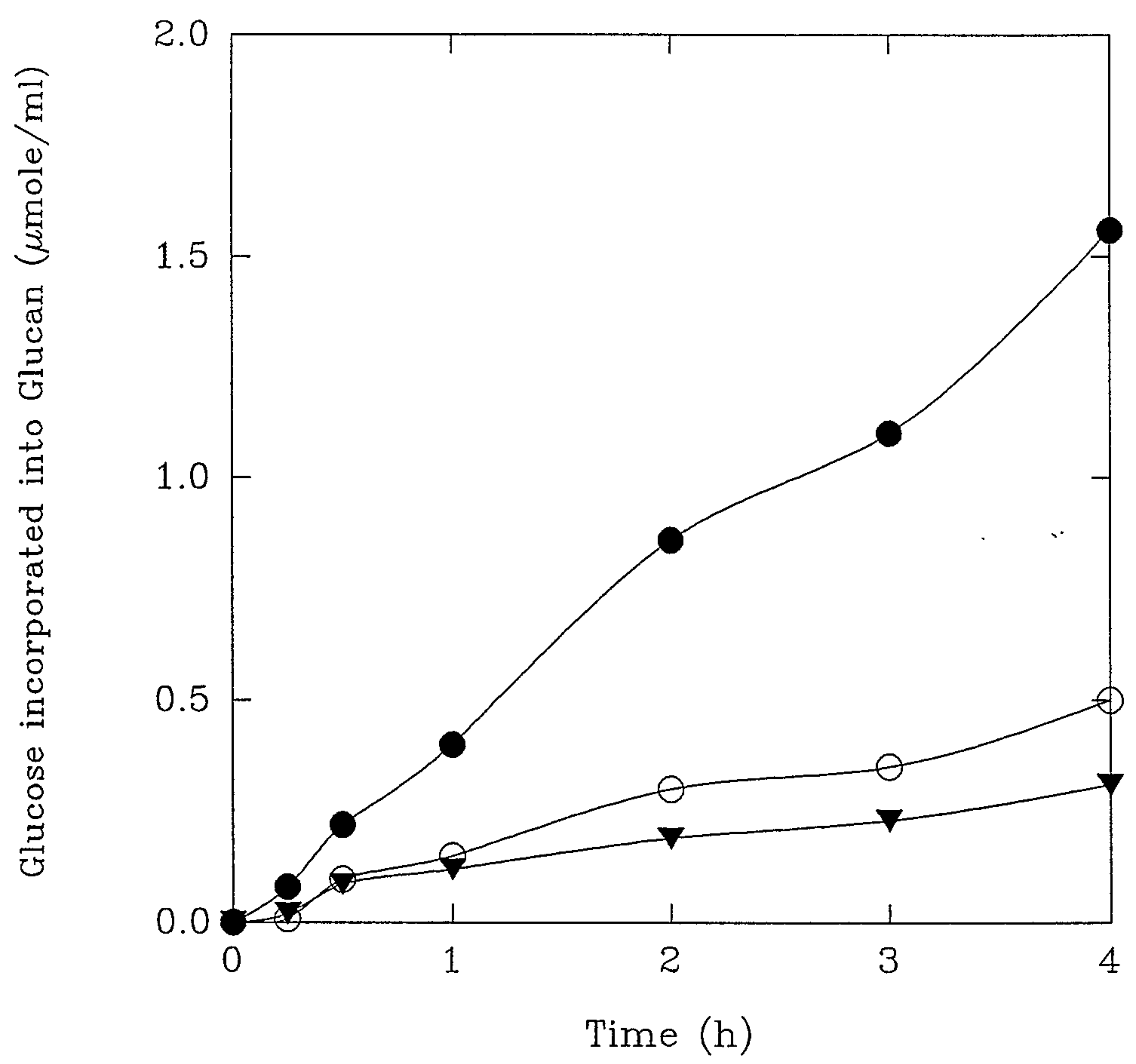


Fig. 8.8. Priming effectiveness of freeze-dried glucan produced by GtfK (○) and dextran T-10 (●) on glucan synthesis by GtfK. The production of glucan without the addition of exogenous primer by GtfK is also shown (▼).



by GtfM and the glucan produced by GtfK are essentially composed of α -1,6-linked glucosyl residues, with no evidence of α -1,3-branches (Chapter 7). Synthetic glucans of this type have previously been reported to activate the primer-dependent GTF-I activity of *S. sobrinus* (Hare *et al.*, 1978). The soluble intermediates noted in the reaction of GtfJ with the GtfK glucan suggest that this glucan is much larger than dextran T-10 and the glucan produced by GtfM where no soluble intermediates were detected. Insolubility of the glucan is due to the ratio of α -1,3 linked to α -1,6-linked glucosyl residues (Walker, 1978).

Although the glucans produced by GtfK and GtfM were acceptors for glucan synthesis by GtfJ, only the glucan synthesized by GtfM is an efficient acceptor for GtfK activity. Although dextran synthesis by primer-dependent GTF-I activities has been studied in some detail, little work has been done on determining the mechanism for glucan synthesis by primer-dependent GTF-S activities. Early experiments with a mixed GTF preparation from *S. sobrinus* indicated that α -1,6-linked glucosyl residues were formed on the non-reducing ends of isomaltosaccharides (Walker, 1973). If chain lengthening does occur in this manner the difference between acceptor efficiency of the glucans produced by the GtfM and GtfK enzymes may be due to the length of the glucan, and thus the number of non-reducing ends available for chain growth by GtfK. The slight increase in the rate of glucan synthesis by GtfK in the presence of its own glucan (Fig. 8.8) might have been the result of some structural change such as the formation of branch linkages on the linear glucan by GtfK, improving its acceptor efficiency.

Unexpectedly, considering it is insoluble, glucan produced by GtfL was also a limited acceptor for the primer-dependent GTFs. This glucan has large percentages of both α -1,6 and α -1,3 linked glucosyl residues (Chapter 7). As it is able to act as an acceptor, this would suggest that the lengths of α -1,6 linked glucosyl residues are of a sufficiently unbranched structure to activate the primer-dependent GTF-I activity. Glucan production by GtfJ was complete in 1h the presence of glucan produced by GtfL. The glucan acted as a better acceptor for GtfK, which may indicate a branched structure with a number of non-reducing ends available for chain lengthening. Both soluble and insoluble glucan was

produced by GtfK in the presence of the glucan synthesized by GtfL (Fig. 8.6B). As more soluble glucan was detected than would be expected through simple autopolymerization by GtfK this suggests the glucan produced by GtfL was solubilized by the addition of α -1,6-linked glucosyl residues by GtfK since the solubility of glucans is dependent on the proportion of α -1,3- and α -1,6-linked glucosyl residues (Walker, 1978).

CHAPTER 9

DISCUSSION

S. salivarius is the most abundant streptococcal species in the human oral cavity, becoming established 24h after birth. *S. salivarius* preferentially colonizes epithelial cells and is usually found on the dorsum of the tongue and the mucosal surfaces of the cheek as well as in saliva (Hamada and Slade, 1980). It is, however, found on the tooth surface, especially during early plaque development and following repeated ingestion of sucrose (Carlsson, 1968). Animal studies with both hamsters and gnotobiotic mice have resulted in many strains being classified into the high-caries category, together with *S. mutans* (Krasse and Carlsson, 1970; Drucker *et al.*, 1984). Both organisms are able to produce a low pH (Drucker *et al.*, 1984), and many strains produce extracellular GTFs that form glucans from sucrose. Nevertheless, *S. salivarius* is generally regarded as a poor colonizer of the tooth surface (van Houte, 1976) and therefore its role in dental caries is not clear. However, one of the main sources of GTF in saliva and in the acquired pellicle is believed to be from *S. salivarius* that is resident on the dorsum of the tongue (Rölla *et al.*, 1983; Scheie and Rölla, 1986). GTFs incorporated in the pellicle are known to be active and to form glucans to which streptococci, such as *S. mutans*, are able to adhere (Shilling and Bowen, 1992). Thus GTFs produced by *S. salivarius* at sites distant from the tooth surface may aid in the initial attachment or entrapment of other oral species to newly erupted tooth surfaces or to tooth surfaces following prophylaxis. It has also been suggested that elimination of *S. mutans* from human plaque by immunization may allow *S. salivarius* to colonize the tooth surface and cause dental caries (Drucker *et al.*, 1984).

S. salivarius ATCC 25975 produces at least four GTFs, which have been cloned and expressed in *E. coli* (Pitty *et al.*, 1989; this study; Table 9.1). The *gtfJ* and *gtfK* genes encoding primer-dependent activities have previously been sequenced (Giffard *et al.*, 1991; Giffard *et al.*, 1993).

The genes encoding the enzymes, GtfJ and GtfK, lie next to each other on the *S. salivarius* chromosome, and are transcribed in the same direction (Giffard

Table 9.1. PROPERTIES OF THE GTFs OF STREPTOCOCCUS SALIVARIUS ATCC 25975

GTF	M_r	pI	pH (Max Activity)	K_m (SUCROSE)* -T10 +T10	ENZYME TYPE	GLUCAN**	PRIMER	CONTIGUOUS <i>gtf</i> GENES
GtfJ	168 000	5.1	5.6	- 20mM	Primer- dependent	90% α -1,3- (insoluble)	T10,M>L>K	<i>gtfK</i>
GtfK	176 000	5.5	6.4	7mM 8mM	Primer- stimulated	100% α -1,6- (soluble)	T10>M>L Not K	<i>gtfJ</i>
GtfL	157 000	5.2	5.2	17mM 14mM	Primer- independent	50% α -1,6- 50% α -1,6- (insoluble)	Inhibited	None
GtfM	171 000	6.8	5.6	8mM 8mM	Primer- independent	95% α -1,6- (soluble)	No Effect	None

* Determined at pH 6.0.

** Dextran T10 contains <5% α -1,3-branch points; percentages of linkages in other glucans are only an estimate (Section 7).

et al., 1991). Sequencing upstream of *gtfJ* showed a region with sequence homology to the *gtfJ* and *gtfK* genes suggesting a third *gtf* gene may lie within this locus. However, attempts to find a clone expressing this putative GTF from the *S. salivarius* gene bank in bacteriophage- λ were unsuccessful.

The close proximity of the *gtfJ* and *gtfK* genes as well as results from previous physiological studies suggesting that GTFs are coordinately regulated in *S. salivarius* (Pitty and Jacques, 1987) is circumstantial evidence in favour of the *gtfJ* and *gtfK* genes forming an operon. In order to determine whether the *gtfJ* and *gtfK* genes formed a single transcript, or whether they were transcribed separately, a method for the isolation of RNA from *S. salivarius* was devised (Simpson *et al.*, 1993). This method relied on the lysis of *S. salivarius* with the muramidase, mutanolysin. This enzyme has optimal activity at 60°C in potassium phosphate buffer containing MgSO₄ and CaCl₂. Under these conditions cell lysis was achieved within 5min, allowing undegraded RNA to be isolated by LiCl precipitation. The development of a procedure for the isolation of RNA from *S. salivarius* ATCC 25975 allowed the transcriptional analysis of the *gtfJK* locus. Both Northern hybridizations and RNase protection assay results indicated that the two genes were transcribed separately, with no transcriptional readthrough.

The *gtfT* and *gtfU* genes of *S. sobrinus* OMZ176 and the *gtfB* and *gtfC* genes of *S. mutans* GS5 are also arranged in tandem (Hanada *et al.*, 1993; Shiroza *et al.*, 1987). In the sequence analysis of the *gtfC* gene a promoter sequence was not identified, leading to the assumption that there was a single promoter for the *gtfBC* locus (Ueda *et al.*, 1988). However, Northern blot analysis has also established that the *gtfB* and *gtfC* genes do not form an operon (Kuramitsu *et al.*, 1991). In light of this evidence it would thus seem likely that this is also the case for the *gtfT* and *gtfU* genes of *S. sobrinus*. GtfT is a primer-independent GTF-S producing high molecular weight glucan (Hanada *et al.*, 1991). The nature of the GTF encoded by *gtfU* has not been reported, although sequence homology with the GtfS of *S. downei*, a primer-independent GTF-S producing oligosaccharides, suggests it may be a similar enzyme (Hanada *et al.*, 1993). If this is indeed the case it may be that these two primer-independent GTF-Ss are those described by Walker *et al.* (1990) which appear to be co-ordinately regulated in media

containing Tween 80. Thus unlike the *gtfJK* and *gtfBC* loci the *gtfTU* genes may, in fact, be under the same regulatory control and form an operon.

The four recombinant GTF activities of *S. salivarius* expressed in *E. coli* differ in regard to their requirement for primer dextran and the solubility of their glucan product with the GtfJ and GtfK enzymes being previously classified as primer-independent (Pitty *et al.*, 1989). Previous studies have suggested the presence of α -1,4-linked glucosyl residues in the glucans from *S. salivarius* (Eifuku *et al.*, 1989; Sato and Inoue, 1991). The glucans produced by the four recombinant GTFs of *S. salivarius* ATCC 25975, however, do not contain any such linkages. This may indicate that there are further uncloned GTFs produced by *S. salivarius*, or alternatively that these original analyses were incorrect.

GtfJ produces an insoluble, essentially α -1,3-linked glucan. This enzyme is similar to previously described GTF-Is, including those of *S. sobrinus* and *S. downei*, which have also been cloned and sequenced (Gilpin *et al.*, 1985; Ferretti *et al.*, 1987; Abo *et al.*, 1991). Although GtfJ was able to produce glucan in a primer-independent manner over an extended period (up to 5 days) it is essentially a primer-dependent enzyme. GtfJ is primed at least to some degree by the glucans produced by the three other known GTFs of *S. salivarius* (Table 9.1).

Primer-dependent GTF-Is depend upon the presence of soluble glucans for polymer-forming activity. They form branches where stretches of α -1,6-linked glucosyl residues occur within these primer dextrans (Walker and Schuerch, 1986). *In vivo* these soluble glucans are thought to be provided by the GTF-S enzymes, which may or may not be primer-dependent. It has been shown that the glucan produced by the GTF-I of *S. sobrinus* OMZ 176 in the presence of one of its GTF-S enzymes is adherent, whereas the glucan produced by the GTF-I in the presence of exogenous glucan could not adhere to glass (Inoue *et al.*, 1982). This suggests that insoluble adhesive glucan is synthesized by the concerted action of the two GTFs.

GtfK can produce significant amounts of soluble glucan in the absence of primer. The addition of dextran, however, strongly stimulates activity. These results indicate that the enzyme should be termed 'primer-stimulated' rather than

'primer-dependent'. Primer stimulation has also been noted for the primer-dependent GTF-S of *S. sobrinus* where the enzyme was described as 'incompletely primer-dependent' (Hanada and Takehara, 1991). The glucan produced by GtfK in the presence or absence of dextran T-10 is composed of α -1,6-glucosyl residues, with no evidence of branch points or other linkages. It therefore differs from the primer-dependent GTF-S of *S. sobrinus* which is a highly branched glucan (McCabe, 1985; Taylor *et al.*, 1990). The glucan formed by GtfK is an acceptor for primer-dependent glucan synthesis by GtfJ. This result is consistent with a previous finding that chemically-synthesised unbranched α -1,6-linked glucan (with a degree of polymerization of 150) is an efficient acceptor for the primer-dependent GTF-I activity of *S. sobrinus* (Hare *et al.*, 1978). In contrast, the primer-dependent GTF-S of *S. sobrinus* has been reported to be a poor enhancer of its own GTF-I (McCabe, 1985), a fact consistent with the highly branched nature of the glucan produced by this GTF-S.

Glucan synthesis by GtfJ in the presence of the glucan formed by GtfK led to the production of soluble intermediates before the appearance of insoluble glucan. This suggests that the glucan produced by GtfK is much larger than dextran T-10, as solubility is dependent on the proportion of α -1,6- and α -1,3-glucosyl residues, and the degree of branching of the final glucan product (Takehara *et al.*, 1992).

The glucan produced by GtfK was a poor acceptor for further glucan synthesis by GtfK. Although dextran synthesis by GTF-Is has been studied in some detail and a mechanism elucidated, similar studies has not been carried out with the 'primer-dependent' GTF-S activities. If chain extension occurs through non-reducing ends, the presence of branch points would provide a number of positions for chain growth. Formation of branch points on the linear glucan would result in improved primer efficiency. The results of this study are consistent with this notion, for the rate of glucan synthesis by GtfK in the presence of its own glucan increased, if only poorly, with time.

The other two cloned GTFs of *S. salivarius* are primer-independent, the GtfL producing an insoluble glucan and GtfM producing a soluble glucan. There was no evidence that the genes encoding these activities formed part of the *gtfJK*

locus or formed their own locus.

GtfL appears unique to *S. salivarius* with no reports of similar activities from other oral streptococci. Under the standard assay conditions used (100mM potassium phosphate buffer pH 6.0) GtfL was strongly inhibited by dextran T-10, and apparently by its own product, resulting in only low levels of glucan production. Inhibition of glucan synthesis by low levels of dextran have not been described for other GTFs of oral streptococci, although insoluble glucan production, either by *S. sobrinus* grown in Tween 80 or by *S. salivarius* grown under a variety of growth conditions, has been shown to be inhibited by relatively low concentrations of dextran ($>50\mu\text{g/ml}$; Wittenberger *et al.*, 1978). Dextran inhibition of GtfL, however, was not apparent when assayed at its optimal pH for activity (pH 5.2). This pH corresponded to the enzyme's pI, suggesting that aggregation of the enzyme, due to its lack of charge, may be responsible for this change in the enzyme's properties. Aggregation has been suggested to be the cause of GTF-S enzymes producing insoluble glucan when in high salt conditions, or in the presence of hydrogen peroxide (Mukasa *et al.*, 1979; McAlister *et al.*, 1989b).

The highly insoluble glucan synthesised by GtfL consisted of high percentages of both α -1,6- and α -1,3-linked glucosyl residues, though it was not known whether the glucan was adhesive. Insoluble adherent glucan is generally thought to be the result of the combined action of GTF-I and GTF-S type enzymes forming a glucan with a highly branched tree-like structure with chains of α -1,3 and α -1,6-linked glucosyl residues (Hare *et al.*, 1978). Insolubility is due to the linear chains of α -1,3-linked glucosyl residues, with the α -1,6-chains related to the adhesiveness of the glucan (Ebisu and Misaki, 1974). The glucan produced by a similar GTF to GtfL from another *S. salivarius* isolate from the human mouth was, however, able to agglutinate *S. sobrinus* (Sato and Inoue, 1991).

The glucan produced by GtfL exhibited limited acceptor activity for both GtfJ and GtfK, which was an unexpected result as adsorption of enzymes onto insoluble glucan is generally believed to lead to their inactivation (Walker, 1978). Both α -1,3-linked glucan and insoluble glucan produced by *S. sobrinus* failed to stimulate glucan synthesis by the primer-dependent GTF-S activity of *S. sobrinus*

(Hare *et al.*, 1978). Furthermore, an α -1,3-linked glucan, made soluble by the introduction of carboxymethyl groups, did not stimulate the GTF-I of *S. sobrinus* OMZ 176 (Walker and Schuerch, 1986). Thus the stimulation of GtfJ and GtfK by the glucan produced by GtfL is probably due to stretches of α -1,6-linked glucosyl residues present in this glucan. These sequences may remain 'dissolved' in water despite their attachment to the α -1,3-linked sequences (Walker, 1978). The synthesis of α -1,6-linked glucosyl residues by GtfK on the glucan produced by GtfL may have led to the solubilization of the glucan.

GtfM produced a soluble glucan of essentially α -1,6-linked glucosyl residues. The addition of exogenous dextran had little effect on stimulating GtfM activity. The glucan produced by GtfM was an efficient acceptor for both GtfJ and GtfK. Primer-independent GTF-S enzymes have been reported from both *S. sobrinus* and *S. downei* (Takehara *et al.*, 1984; McCabe, 1985; Gilpin *et al.*, 1985; Russell *et al.*, 1990; Taylor *et al.*, 1990; Hanada *et al.*, 1993a). These produce either high molecular weight branched glucan similar to the glucan of GtfM or linear oligo-isomaltosaccharides. A mutant of *S. downei* that does not produce primer-independent GtfS and therefore does not synthesize oligo-isomaltosaccharides from sucrose can no longer synthesize water-insoluble glucan and has a decreased ability to adhere to glass (Gilmore *et al.*, 1993). This is the case despite the fact that two other GTF-Ss are secreted by *S. downei* (Gilmore *et al.*, 1993). While a similar enzyme to GtfS is produced by *S. sobrinus* it does not synthesize an effective primer for its own primer-dependent GTF-I and GTF-S, but rather appears to reduce the molecular size of the glucan produced by the other primer-independent GTF-S of *S. sobrinus* (Yamashita *et al.*, 1989).

The analyses of the various kinetic properties of the four recombinant GTFs of *S. salivarius* indicated that both the K_m for sucrose did not vary greatly and that the optimal pH was in the range of pH 5.2 to pH 6.4. This would suggest that all four GTFs function under similar conditions in the oral cavity.

Little is known about the regulation of GTFs of *S. salivarius* or other oral streptococci. Whether the GtfJ, GtfK, GtfL and GtfM are all expressed concurrently is unknown, although Northern analysis indicates that the activities are all expressed when cells are grown in the presence of Tween 80. Studies by

Jacques and coworkers have suggested that phase changes in the membrane lipids may affect GTF production in *S. salivarius* (Jacques, 1985; Jacques *et al.*, 1985; Markevics *et al.*, 1987; Pitty and Jacques, 1987). Buchan and Jenkinson (1990) suggested that in addition to membrane modulation, Tween 80 may also have a direct inhibitory or stimulatory effect on GTF synthesis at the transcriptional level. This appears to be the case in *S. sobrinus* where growth of cells in the presence of Tween 80 results in the secretion of two previously undetected primer-independent GTF-S activities, as well as increased amounts of GTF-I (Walker *et al.*, 1990). However this may not be the case in *S. salivarius* as growth of cells in the presence of Tween 80 does not alter the effect of primer dextran on insoluble glucan synthesis. This contrasts to the dramatic change from stimulation to inhibition noted with the GTFs of *S. sobrinus*, depending upon whether the surfactant was present or absent from the growth medium (Wittenberger *et al.*, 1978).

In another study, both growth rate and the presence of sucrose were found to affect the transcriptional activity of the *gtfB* promoter of *S. mutans* (Wexler *et al.*, 1993). Total secreted GTF activity, however, did not always parallel *gtfB* transcriptional activity, suggesting that the other two GTFs of *S. mutans* may be regulated in a different manner. The results, however, clearly suggest that environmental conditions influence the expression of GTFs. As individual GTF activities are difficult to monitor in the culture fluid, future determination of the levels of the mRNA transcripts of specific GTFs should allow aspects of regulation to be studied in more detail as well as allowing a comparison to be made of the expression of different GTFs within a given streptococcal species.

The deduced amino acid sequences of GtfL and GtfM determined in this study could be readily aligned with the nine previously sequenced GTFs. The results indicated that both GtfL and GtfM proteins contained typical streptococcal signal sequences for which possible cleavage sites could be defined according to von Heijne's '-3, -1' rule (von Heijne, 1983). These signal sequences, like all leader peptides, contained a basic N-terminal polar region followed by a hydrophobic core and a polar C-terminal region. The GtfL signal sequence was 35 amino acids in length, while that of GtfM was 38 amino acids long. The signal

sequence of all eleven known GTF sequences contained a high number of identical and conserved amino acids (Fig. 6.9).

The signal sequences were followed by poorly aligned regions of approximately 180-200 amino acids. The role of this region is not known, though N-terminal deletions of the GtfL (this study) and the GtfJ of *S. salivarius* (Giffard and Jacques, unpublished observations) as well as the GtfI of *S. sobrinus* (Abo *et al.*, 1991) that extend to different degrees into this region have no apparent effect on enzyme activity.

The region surrounding the putative active sites of GtfL and GtfM were highly conserved. This region has been designated the 'catalytic domain' and is some 900 odd amino acids in length. Work by Mooser and coworkers (Mooser and Iwaoka, 1989; Mooser *et al.*, 1991) have identified an active site aspartic acid residue in this region following the isolation from both the GTF-I and the GTF-S of *S. sobrinus* of covalent glucosyl-enzyme complexes. Mass spectrometric analyses of these enzymes partially digested with pepsin, indicate that the Asp-6 within the GTF-I nonapeptide, Asp-Ser-Ile-Arg-Val-Asp-Ala-Val-Asp, was the carboxyl group forming the covalent glucosyl-enzyme intermediate (Mooser *et al.*, 1991). This was confirmed by site-directed mutagenesis of this aspartic acid residue (Asp-451) in the GtfB of *S. mutans*, since its replacement results in no detectable sucrase or polymer-forming activities, whilst the enzyme retains dextran-binding activity (Kato *et al.*, 1992). Comparison of the active sites of all eleven sequenced GTFs indicated that the amino acid sequences possessed a high level of identity surrounding the conserved aspartic acid residue, with essentially only conservative substitutions (Fig. 6.10).

Catalysis by GTFs, however, also requires the participation of a general proton donor to facilitate fructose departure. The photooxidation studies of Koga and Inoue (1981) have suggested the involvement of histidine residues in GTF activity. The GTF of *S. mutans* AHT was completely inactivated under conditions where only histidine residues were modified, strongly suggesting that the imidazole portion of histidine functioned as part of the GTF active site. Alignment of the deduced amino acid sequences revealed only three conserved histidine residues in the eleven GTFs. These histidines bracket two highly conserved aspartic acid

rich regions, one of which contains the active site aspartic acid residue. It is perhaps of interest that the first of these conserved histidine residues lies within a chimeric protein composed of a fifteen amino acid peptide from GtfB (342-356) fused to the B subunit of cholera toxin, antisera to which inhibits water-insoluble glucan synthesis by *S. mutans* (Dertzbaugh and Macrina, 1990). It would be of interest to determine by site-directed mutagenesis what effect replacing these three histidine residues has on the sucrase and polymer-forming activity of GTFs.

Other regions within this conserved area are also thought to have an important biological function. Genetic variations among *gtfB* and *gtfC* genes of *S. mutans* are conservative and do not affect the encoded amino acids (Chia *et al.*, 1991; Chia *et al.*, 1993). Monoclonal antibody to a sub-sequence peptide from the 'catalytic' domain of GtfC (435-453) inhibited GtfC activity, but had no effect on GtfD despite the latter enzymes having the same peptide sequence (Chia *et al.*, 1993). Interestingly, antisera against peptides derived from the active-site sequence do not inhibit the GtfI and GtfS activities of *S. downei*, presumably due to the enzyme's conformation (Cope and Mooser, 1993).

Construction of a phylogenetic tree from the conserved 'catalytic' domain indicated that if one were to exclude the grouping of GtfB/GtfC and the GtfIs of *S. sobrinus* and *S. downei* (which are identical over much of their length), there was little clustering of enzymes with similar activities. This is a similar result to that previously reported by Giffard *et al.* (1993) who found no clustering of GTF-I or GTF-S type enzymes. The two primer-independent GTFs of *S. salivarius* (GtfL and GtfM) grouped with the primer-dependent GTF-S of *S. mutans* (GtfD), although they were not closely related. The primer-dependent GTFs of *S. salivarius* (GtfJ and GtfK), also did not group with primer-dependent activities from other streptococci.

In contrast to the 'catalytic' domain, the C-terminal glucan-binding domains of the GTFs did not align. This was not surprising as this region of the proteins contains a large number of repeating units that have undergone a number of independent duplications and arrangements (Giffard and Jacques, 1993; 1994).

These repeating units, which have previously been divided into four

different classes (A,B,C,D) on the basis of sequence similarity, have been shown to be composed of a common structural element, designated the 'YG' repeat (Giffard and Jacques, 1994). The A,B,C, and D classes of repeats are thought to arise from duplication and divergence of a number of 'YG' repeats (Giffard and Jacques, 1994), and thus should contain a number of the smaller 'YG' motifs. 'YG' repeats could be identified in the C-terminus of GtfL and GtfM, the former having 15 full and 1 partial and the latter 21 repeats. Phylogenetic trees of the 'YG' repeats of GtfL and GtfM were constructed to determine if there were any evidence of duplication events. Although there was no evidence of clustering between the repeats of GtfM, the phylogenetic tree of GtfL had three clusters of four or five repeating units. Closer analysis of these clusters suggested a triplet of sequential 'YG' repeats had been duplicated to give four series of these repeats. Within these triple repeats, 45% of the amino acids were conserved or identical. Duplication of a triplet of 'YG' repeats was also suggested in a previous analysis of the GtfJ and GtfK proteins (Giffard and Jacques, 1994). Duplication events of this kind may result in increased receptor binding or may enable the enzymes to avoid immune responses through sequence variation as a result of recombination events (Giffard and Jacques, 1994). If the latter were the case this would have implications in the design of anti-caries vaccines based on these repeats, where peptides from this repeat region have already been tested as immunogens (Smith *et al.*, 1993).

Although it is clear that GTFs have two distinct domains responsible for the sucrase and glucan-binding activities of the enzymes it is not known what regions of the protein are responsible for primer-dependence and the structure of the glucan produced from sucrose. Sequence analysis gave no indication of any regions unique to primer-dependent or primer-independent activities. The four enzymes cloned from *S. salivarius* all have different activities and represent all the possible combinations of primer-dependence and product solubility. It would thus be of particular interest to carry out domain swapping experiments, where the catalytic and glucan-binding domains of the different enzymes are ligated to form hybrid enzymes. If these constructs exhibit GTF activity, the domains responsible for primer-dependence and glucan structure could then be determined. The

possibility of constructing such hybrid proteins is made easier by the fact that the *gtfJ* gene has a unique *Bgl*III site at nucleotide position 2963, corresponding to amino acid 987. This amino acid lies in the highly conserved region preceding the glucan-binding domain. *Bgl*III sites can be introduced into the other three genes by site-directed mutagenesis, with a maximum 2 base pair change. This site has already been introduced into *gtfL* with no apparent change in enzyme activity. Attempts to carry out domain swapping experiments between GtfL and GtfJ however, have thus far proved unsuccessful, with no recombinants having been isolated (Simpson and Jacques, unpublished observations).

The extent to which this strategy will be successful depends upon the structure of the protein. There has been no structural analysis of any GTF reported thus far, although Mooser *et al.* (1991) has suggested that separate sucrose- and glucan-binding domains together with the absence of a reverse reaction are consistent with a sucrose-induced conformational change within the GTFs. Where separate binding domains exist in other enzymes, a functional active site requires perfect alignment of the domains for enzyme catalysis to occur. If this were the case for the GTFs, domain swapping may result in a protein whose domains no longer orientate to form an active site. This may account for the results obtained with the hybrid enzymes created using the primer-independent GtfB and primer-dependent GtfD of *S. mutans* (Nakano and Kuramitsu, 1992). The activities of the hybrid enzymes were all extremely low, raising doubts about the conclusions that could be drawn from these experiments. What was found, however, was that all active hybrids produced insoluble glucan in the absence of dextran, with glucan synthesis by some hybrids being stimulated by the addition of dextran T-10. This suggested that primer-dependence and glucan structure were influenced by the glucan-binding domain, but not uniquely.

Clearly even if a result is obtained from domain swapping experiments further analysis of smaller regions of amino acids would be required. The proteins are large and site-directed mutagenesis represents a formidable task. Structural analysis of the protein by X-ray crystallography or other means may prove a better approach in the near future.

APPENDIX 1

PREPARATION OF SEMI-DEFINED MEDIA (SDM)

SDM was prepared by mixing the following constituents in the proportions shown:

Salt solution ¹	10 ml
1M Potassium Phosphate Buffer pH7.6	10 ml
Vitamin solution ²	2 ml
0.2% (w/v) Adenine and 0.2% (w/v) Guanine solution (HCl to effect solution)	1 ml
0.2% (w/v) Uracil solution (NaOH to effect solution)	1 ml
5% (w/v) hydrolysed casein	10 ml
H ₂ O	<u>56 ml</u>
Final volume	90 ml

The above solution was autoclaved at 15lbs/in² for 20min, cooled and the following filter sterilized solutions added prior to use:

- (a) 5 ml of 1.1% (w/v) cysteine HCl
- (b) 2 ml of 22.52% (w/v) glucose, 1 ml 5% (w/v) NaHCO₃, 2 ml H₂O

¹ Salt Solution

(NH ₄) ₂ SO ₄	10 g
MgSO ₄ .7H ₂ O	2 g
FeSO ₄ .7H ₂ O	0.4 g
MnSO ₄ .H ₂ O	0.13 g
NaAcetate.3H ₂ O	3 g
H ₂ O	1 litre

Allow Fe complexes to precipitate and use after filtering.

² Vitamin Solution

Thiamine-HCl	0.20 g
Pyridoxamine.2HCl	0.05 g
Ca-pantothenate	0.20 g
Riboflavin	0.20 g
Nicotinamide	0.10 g
<i>p</i> -aminobenzoic acid	0.01 g
Biotin	0.005 g
Folic acid	0.005 g
H ₂ O	1 litre

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THESIS EMENDATIONS

1. Corrections to Text, Figures and Tables

Page	Line	As Presented	Correction
Preface	3	that	thank
xi	13	cytodine	cytidine
xi	15	deoxycytodine	deoxycytidine
3	16	Binder <i>et. al.</i> , 1983	Binder and Robyt, 1983
7	11	streptococci	<i>Streptococcus</i>
8	1	Hanada <i>et. al.</i> , 1993	Hanada <i>et. al.</i> , 1993a
15	16	radiolabeled	radiolabelled
16	24	<i>S. sanguis</i> ATCC 10558	<i>S. gordonii</i> ATCC 10558
21	7	1978	1977
21	14	Curtis	Curtiss
23	29	McAlister <i>et. al.</i> , 1989	McAlister <i>et. al.</i> , 1989b
28	2	Dibdin <i>et. al.</i> , 1981	Dibdin, 1981
28	12	<i>Actinomyces viscosus</i>	<i>Actinomyces naeslundii</i>
28	13	1975; 1975a	1975a; 1975b
29	29	<i>A. viscosus</i>	<i>A. naeslundii</i>
32	4	Rundegran	Rundegren
40	8	precipitant	precipitate
43	7	16 000	16 000g
59	9	affect	effect
59	21	identified a putative	identified putative
64	28	was	were
66	12	20 000 thousand	20 000
72	25	Russell <i>et. al.</i> , 1985	Russell <i>et. al.</i> , 1985a
73	17	Na ₂ PO ₄	Na ₂ HPO ₄
75	2	absorbency	absorbance
84	12	Fig. 5.384	Fig. 5.3
89	Fig 5.6	116	116 000
89	Fig 5.6	170	170 000
108	Fig 6.3	1062	1052
108	Fig 6.3	1008	1098
135	Fig 6.10	GtfC, 482-491	GtfC, 472-480
135	Fig 6.10	GtfD, 467-475	GtfD, 460-468
135	Fig 6.10	<i>S. downei</i> GtfI, 543-461	<i>S. downei</i> GtfI, 448-456
135	Fig 6.10	GtfS, 443-451	GtfS, 432-440.
136	18	independent	dependent
138	Fig 6.11	GtfD, 166-1069	GtfD, 178-1069
138	Fig 6.11	GtfI (dn), 168-1046	GtfI (dn), 164-1041
138	Fig 6.11	GtfS (dn), 156-1026	GtfI (dn), 154-1023.
144	20	1987	1987a
178	19	concentraion	concentration
184	26	Yamashita <i>et. al.</i> , 1988	Yamashita <i>et. al.</i> , 1988b
205	Table 9.1	50% α -1,6-, 50% α -1,6-	50% α -1,3-, 50% α -1,6-
213	12	Chia <i>et. al.</i> , 1993	Chia <i>et. al.</i> , 1993a
213	14	Chia <i>et. al.</i> , 1993	Chia <i>et. al.</i> , 1993b

THESIS EMENDATIONS

2. Corrections to Bibliography

Page	Line	Alteration
217	6	'Adise-Opuku' should read 'Aduse-Opuku'
218	21	'1969' should read '1968'
224	6	'in press.' should read '73:1133-1141.'
225	5	The citation should read 'Haisman RJ, Jenkinson HF (1991). Mutants of <i>Streptococcus gordonii</i> Challis over-producing glucosyltransferase. <i>J. Gen. Microbiol.</i> 137:483-489.'
227	19	'pp 2-32' should read 'pp 2-32. Information Retrieval Inc., Washington'
228	1	'Curtis' should read 'Curtiss'
230	11	'Koba' should read 'Kobs'
235	16	'1974' should read '1974a'
235	19	'1974' should read '1974b'
240	6	Missing reference should read 'Shimamura A (1989). Use of ¹³ C-N.M.R. spectroscopy for the quantitative estimation of 3-O- and 3,6-di-O-substituted D-glucopyranosyl residues in α -D-glucans formed by the D-glucosyltransferases of <i>Streptococcus sobrinus</i> . <i>Carbohydr. Res.</i> 185:239-248.'
242	14	'Doyle, RI' should read 'Doyle, RJ'

