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PHOSPHOROLYTIC SYNTHESES WITH DI-, OLIGO- AND MULTIFUNCTIONAL PRIMERS*

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ABSTRACT

Studies on preparation of linear, star-, and comb-shaped polymers carrying amylose chains of uniform length grown by synthesis with potato phosphorylase are reported. Binding of malto-oligosaccharide primers (A) to different supports (B) was performed by the reaction of aldonolactone or *N*-(2-aminoethyl)aldonamide derivatives of malto-oligosaccharides with carriers respectively containing NH_2 or CO_2H groups. Synthetic products were converted into the tricarbanilate derivatives, and their hydrodynamic behavior was studied by light-scattering and viscosity measurements. The molecular-weight distribution was examined by 10-MPa l.c. Phosphorolytic syntheses with difunctional primers, A–B–A [with B = oligo(methylene) or oligo(oxyethylene)], proceed in the same way as with monofunctional primers. Both chain ends are elongated equally to amyloses of uniform length. Oligofunctional primers, $(A)_xB$ with $x = 3$, 4, and 6, lead to a mixture of components differing in the number of long branches, but with the amylose chains all having approximately uniform lengths. Compounds with two branches are preferentially formed. The results are discussed with regard to (*a*) the restrictions of the potato phosphorylase and (*b*) a model in which two primer ends from the oligofunctional substrate molecule are simultaneously bound to the active centers of the phosphorylase dimer. Compared with these densely packed primers, malto-oligomers attached to a linear backbone chain of poly(vinylamine) are attacked more easily. This can be explained by a greater flexibility of the poly(vinylamine) chain.

INTRODUCTION

This work deals with an attempt to obtain regularly shaped, linear and branched polymers carrying amylose chains of uniform length grown by phosphorolytic synthesis. The *in vitro* synthesis, with potato phosphorylase from D-glucosyl phosphate (G-1-P) and a malto-oligomer as a primer, has been shown to

*Linear and Star-Shaped Hybrid Polymers, Part IV. For Part III, see ref. 1

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proceed analogously to living, anionic polymerization[2,3]. Amyloses of a very narrow (Poisson) molecular-weight distribution are formed under the following conditions. (*1*) Simultaneous start for all chains: the primer must have a minimum length of four D-glucosyl residues. (*2*) Simultaneous growth of all chains: the enzyme–substrate complex has to dissociate after each step of addition. (*3*) No change in the number of growing chains: buffered solution and absence of alpha amylase to preclude hydrolytic and enzymic cleavage. (*4*) No chain-termination step: the nonreducing primer end-groups cannot be deactivated chemically, but previous precipitation of amylose from aqueous solution has to be avoided.

Under these conditions, the primer has the same function as has the initiator in anionic polymerizations. The final degree of polymerization (d.p.) can be regulated simply by the ratio of monomer to primer and by the degree of conversion.

Contamination of the primer with maltotriose no longer leads to amyloses of uniform lengths. When using maltotriose as a primer, products are composed of a fairly monodisperse, high-molecular-weight component and a long tail of shorter chains[2,3]. This typical distribution can be explained by kinetics involving a much slower initiation step, as the binding of maltotriose to the enzyme is 1/400th that of maltotetraose. The binding constants of maltotetraose and the next higher oligomers are the same. From these results, it may be concluded that the four terminal residues at the nonreducing chain end fit into the active center of the potato phosphorylase.

This finding prompted us to prepare additionally branched polymers of variable shape. Primers were chemically bound to different carriers by amide linkages[1,4–6]. The procedure involves the reaction of malto-oligomers in the range of d.p. 5–8 having aldonolactone end-groups with compounds containing amino groups, or having amino end-groups [*N*-(2-aminoethyl)aldonamides] with compounds containing carboxyl groups. Depending on the number and arrangement of functional groups, A–B–A block-copolymers, star- and comb-shaped polymers should be obtained from di-, oligo-, and multi-functional primers, respectively. Suitable models for the fine architecture of amylopectin are found in the group having 3–6 primer chains.

In order that the phosphorolytic synthesis might proceed in the expected way, special care was taken, in the course of the derivatization and coupling of malto-oligomers, to prevent hydrolytic degradation and the eventual formation of maltotriose stubs[1].

RESULTS

A–B–A Block-copolymers. — In a first set of experiments, syntheses were carried out with difunctional primers derived from the reaction of malto-oligosaccharide aldonolactones (A) with aliphatic diamines and α,ω-diamino-substituted hexa(oxyethylene)[6,7] (B). All polymerizations were conducted with 4 g of the

monomer G-1-P and with a variable ratio of primer to monomer. The amount of phosphorylase was not changed. The results are shown in Table I.

As derived from the viscosity measurements of the tricarbanilate derivatives, for each series a continuous increase in the degree of polymerization ($d.p._{\eta}$) is found with decreasing ratio of primer to monomer. $D.p._{\eta}$ values for synthetic products from primers with B = $-(CH_2)_6-$ and $-(CH_2)_{12}-$ (see series S6 and S12 in Table I) tend to be higher than the degrees of polymerization calculated from the ratio of monomer to primer and the degree of conversion ($d.p._{calc}$). There could be several reasons for this: *e.g.*, humidity in the sample of primer (a somewhat lower actual primer concentration), or the hydrodynamic dimensions of the A–B–A polymers differing from those of the amylose tricarbanilates.

Only when more closely examining the molecular-weight distribution by liquid chromatography (l.c.) did we realize that our initial products all exhibited a bimodal distribution. (see Fig. 1). As may be seen in Table II, the two components had a molecular weight ratio of 2:1. The average degree of polymerization ($\overline{d.p.}$)

TABLE I

PHOSPHOROLYTIC SYNTHESES WITH DIFUNCTIONAL PRIMERS DERIVED FROM COUPLING OF MALTOHEPTAONOLACTONE TO ALIPHATIC DIAMINES AND α,ω-DIAMINOHEXA(OXYETHYLENE) AS B-BLOCK

Product	*Primer (mg)/ 4 g of G-1-P*	*D p (calc.)*	$[\eta]^a$ *(mL/g)*	$D\,p_{\eta}{}^b$
$B = -(CH_2)_2-$[c]				
S2-1	38.6	335	66	365
S2-2	10.3	950	165	975
$B = -(CH_2)_6-$[c]				
S6-1	109 0	145	42	220
S6-2	66 0	230	62	340
S6-3	46 0	335	89	500
S6-4	31 0	500	120	690
S6-5	21 0	750	160	950
$B = -(CH_2)_{12}-$[c]				
S12-1	113.0	135	38	200
S12-2	68 5	215	53	285
S12-3	48 0	270	70	400
S12-4	32 5	500	118	680
S12-5	21 5	750	151	900
$B = -(CH_2CH_2O-)_6-$[d]				
S6'-1	66 0	300	62	340
S6'-2	31 0	615	112	650
S6'-3	15 0	1270	192	1150
S6'-4	7 5	2580	322	2050

[a]Intrinsic viscosity of the tricarbanilate in 1,4-dioxane (20°) [b]From the viscosity–molecular weight relationship of Sutter and Burchard[8]. [c]Coupling to $H_2N(CH_2)_nNH_2$, with n = 2, 6, and 12. [d]Coupling to $H_2NCH_2CH_2(OCH_2CH_2)_5NH_2$

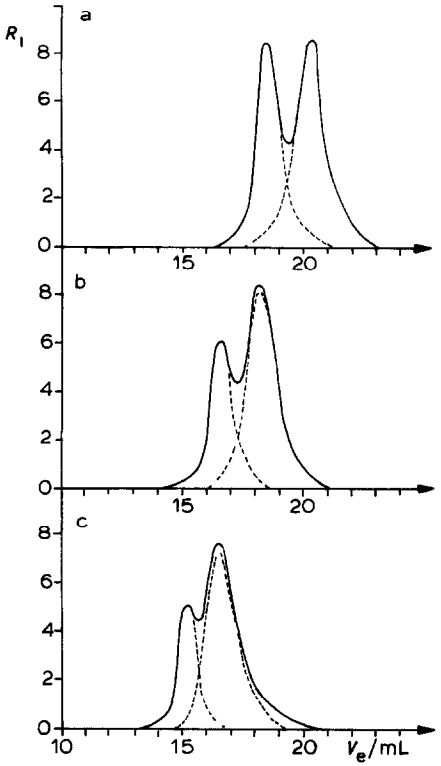

Fig 1 10-MPa l c diagrams of tricarbanilates in THF. [Syntheses from difunctional primers containing A–B–A and A–B polymers (a) S6-1, (b) S6-3, (c) S6-5. (Data from curve analysis, see Table II)]

from l.c. agreed quite well with $d.p._{\eta}$. Thus, the products contained A–B–A and A–B polymers, which seemed to indicate that the phosphorylase is not capable of using both primer ends to equal extents. This was not in agreement with the results of previous kinetics studies[9].

All the primers used so far had been purified at each conversion step by ion-exchange chromatography using poly(styrene) resins. A thorough reinvestigation revealed that this treatment was not sufficient to remove completely the oligosaccharide aldonolactone (A) and the A–B byproduct[1]. In addition, by passage through the strong-acid resin [IR 120 (H^+)], resplitting of amide bonds may occur. A new method elaborated for purification proved an excellent tool[1]. By chromatography using a cross-linked, poly(acrylamide) gel (Bio-Gel P-4 or P-10) containing a small proportion of carboxyl groups, unreacted material and partially substituted products carrying amino or carboxyl groups can easily be removed. This procedure was applied in all further studies.

A–B–A polymers prepared with the pure, difunctional primers now gave uniform products with a narrow molecular-weight-distribution, indicating that the phosphorylase attacks both ends of the primer equally (see Fig. 2). Only when the

TABLE II

COMPONENTS OF POLYMERS FROM FIRST SYNTHESES WITH DIFUNCTIONAL PRIMERS DETERMINED BY 10-MPa L C–G P C

Product[a]	*D.p.*		%		$\overline{D.p.}$ (10-MPa l.c.)	$D.p._{\eta}$	$D.p._{calc}$
	ABA	*AB*	*ABA*	*AB*			
S2-1	600	305	50	50	450	365	335
S6-1	320	155	50	50	230	220	145
S6-2	505	245	45	55	350	340	230
S6-3	750	370	43	57	520	500	335
S6-4	1010	530	42	58	720	690	500
S6-5	1460	770	40	60	1010	950	750
S12-1	285	140	45	55	205	200	135
S12-2	420	210	30	70	290	285	215
S12-3	610	300	30	70	390	400	270
S12-4	970	500	30	70	670	680	500
S12-5	1640	820	30	70	970	900	750
S6'-1	550	280	30	70	355	340	300
S6'-2	1080	570	20	80	655	650	615

[a]For designation of products, see Table I.

primer contained a long poly(oxyethylene) sequence of $\overline{d.p.}$ 45, *i.e.*, S(PEO) in Table III, did the molecular-weight distribution become broader. Apparently, interactions between the protein and the polar poly(oxyethylene) do not allow the enzyme–substrate complex to dissociate as readily as before.

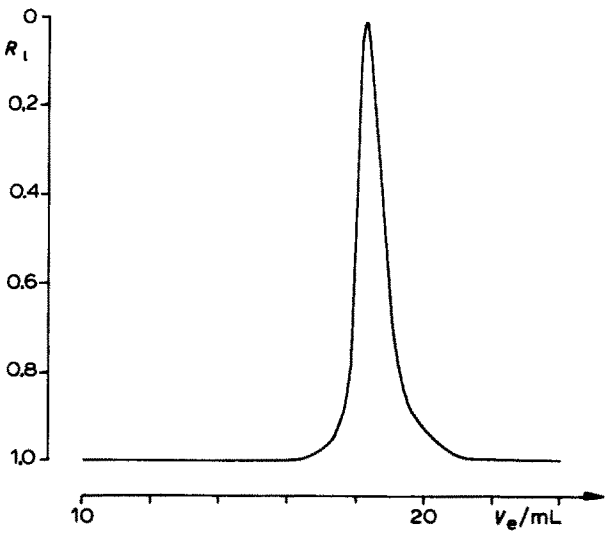

Fig. 2. 10-MPa l c. diagram of A–B–A polymer from pure, difunctional primer (S12-1, see Table III).

TABLE III

RESULTS FROM LIGHT-SCATTERING AND VISCOSITY OF TRICARBANILATED POLYMERS

Product	$M_w \cdot 10^{-5}$ *(g/mol)*	P_w	$[\eta]$ *(mL/g)*	$\langle S^2 \rangle_z^{1/2}$ *(nm)*
A–B–A polymers				
S6-1[a]	2 00	385	78	23 0
S6-2[a]	3 95	760	144	34 6
S6-3[a]	13.00	2505	385	66 5
S6-4[a]	33 00	6350	995[b]	126 0
S12-1[a]	1 60	310	56	18 6
S12-2[a]	3 53	680	128	31 8
S6′-1[a]	31 10	6000	885[b]	123 7
S(PEO)-1[c]	2 93	565	96	25 8
S(PEO)-2[c]	5 13	990	155	41 6
S(PEO)-3[c]	7 00	1350	215	49 0
S(PEO)-4[c]	12 60	2410	305	68 3
Star polymers[d]				
S3*-1	1 77	340	67	19 4
S3*-2	2 50	480	82	25.0
S3*-3	3 62	700	130	30 0
S4*-1	2 53	485	100	23 5
S4*-2	4 90	940	158	36 3
S6*-1	1 03	200	41	14 0
S6*-2	1 42	275	60	19 0
S6*-3	5 20	1000	163	41 8
S6*-4	9 52	1835	275	59 0
Comb polymers[e]				
S(PVA)-1 d s = 0 1	59 00	11330	146	90 5
S(PVA)-2 d s = 0 1	83 30	16000	172	117 0
S(PVA)-3 d.s = 0 3	41 70	8035	246	92 6
S(PVA)-4 d s = 0 3	100 00	19270	290	158 0
S(PVA)-5 d s = 0 6	200 00	38530	272	223 0

[a]Prepared from purified, difunctional primers Designations otherwise as in Table I [b]Intrinsic viscosity extrapolated to zero shear-rate from measurements made with an Ubbelohde viscosimeter [c]Malto-oligosaccharide aldonolactone primer coupled to α,ω-diamino-substituted poly(oxyethylene) (PEO, mol wt 2000, d.p 45) [d]For designations, see Table IV. [e]Malto-oligosaccharide aldonolactone primer coupled to poly(vinylamine) (PVA)

The results from light-scattering and viscosity measurements (first group in Table III) are seen in Figs. 3 and 4. We were interested to know whether this type of A–B–A molecule, having amylose chains stiffened by bulky phenylcarbamate substituents, and held together by a flexible link, could eventually serve as a model of the "once-broken rod", first introduced by Yu and Stockmayer[12]. However, no difference between the molecular-weight dependence of the intrinsic viscosity or the root-mean-square radius of gyration of A–B–A and amylose tricarbanilates (A) was found, indicating that the flexible joint does not affect the chain dimensions to a significant extent. Dielectric relaxation studies clearly revealed a head-to-tail

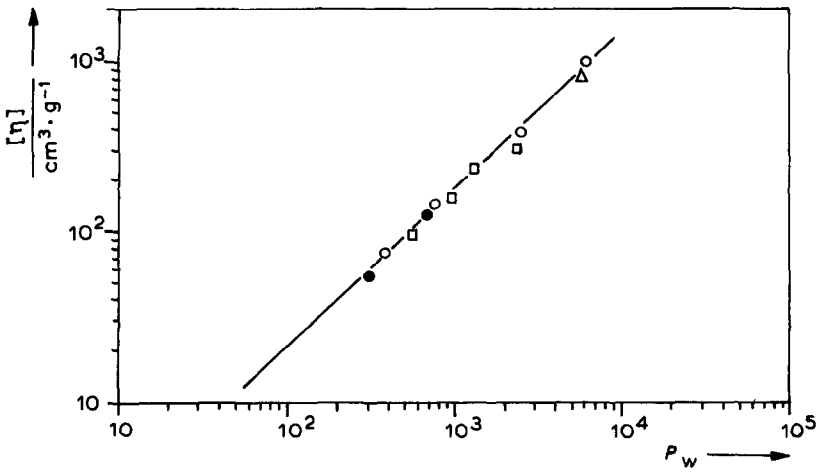

Fig 3 Dependence of the intrinsic viscosity ([η]) on degree of polymerization (P_w) for A–B–A polymers. [Key: B-blocks; (○) –$(CH_2)_6$–, (●) –$(CH_2)_{12}$–, (△) –$(CH_2CH_2O–)_6$–, and (□) –$(CH_2CH_2O–]_{45}$– For data, see Table III Solid line amylose tricarbanilates[8]]

arrangement of the dipoles in the A samples, and a head-to-head arrangement in the A–B–A samples, thus proving the block-copolymeric structure of the latter[11].

Star polymers. — Three and 4-functional primers were prepared from 1,3,5-benzenetriacetic acid and (ethylenedinitrilo)tetraacetic acid as carriers[1,4], and the 6-functional primer from a hexaamino derivative of D-glucitol[1,6,7] (see the Experimental section). The last product was intended to serve as a model of the trichitic, or cluster-like, branched structure suggested for amylopectin[13–15]. A remarkably dense packing of the 6-functional primer with six maltoheptaose side-chains could be directly deduced from g.p.c. on Bio-Gel P-10. The compound (mol. wt. 7500) was eluted in the range of maltohexaose (mol. wt. 990)[1].

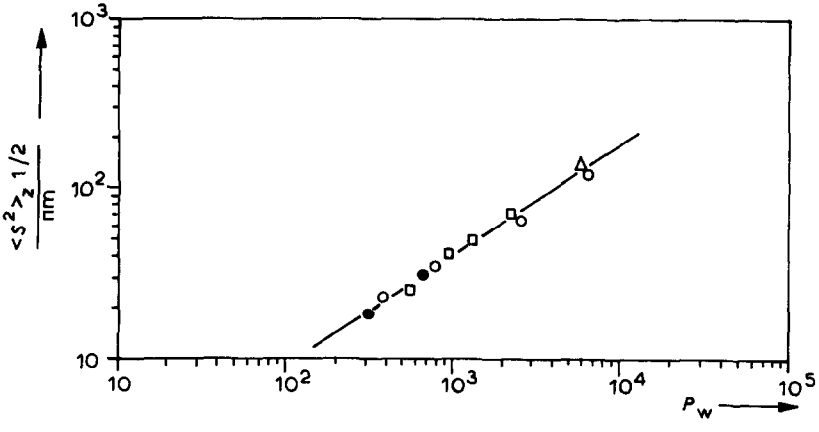

Fig. 4 Dependence of the radius of gyration ($\langle S^2 \rangle_z^{1/2}$) on degree of polymerization (P_w) for A–B–A polymers. [For symbols, see Fig. 3. Solid line, amylose tricarbanilates[10,11].]

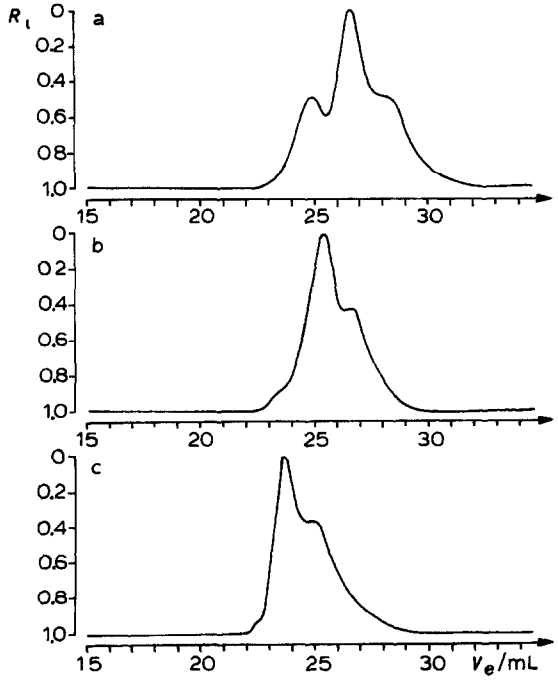

Fig. 5 Syntheses from the 3-functional primer (10-MPa 1 c.). [(a) S3*-1 (70), (b) S3*-2 (40), (c) S3*-3 (20). Primer concentration (mg/4 g of G-1-P) in parentheses.]

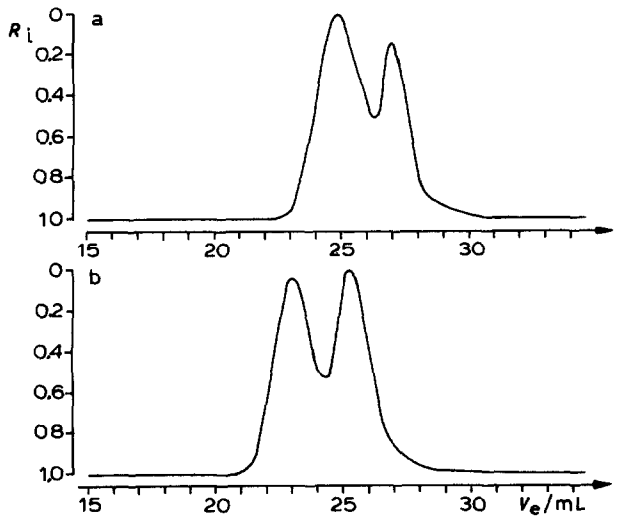

Fig 6. Syntheses from the 4-functional primer (10-MPa 1 c) [(a) S4*-1 (60), (b) S4*-2 (30). Primer concentration (mg/4 of G-1-P) in parentheses.]

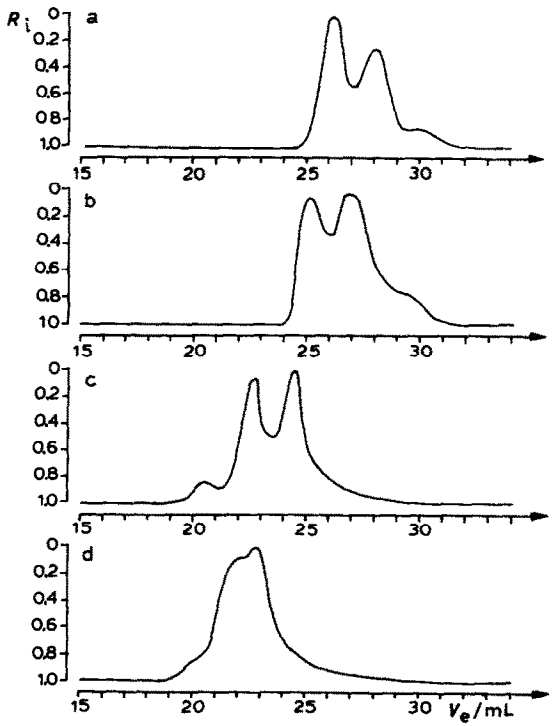

Fig. 7 Syntheses from the 6-functional primer (10-MPa l.c.) [(a) S6*-1 (130), (b) S6*-2 (80), (c) S6*-3 (20), (d) S6*-4 (10) Primer concentration (mg/4 g of G-1-P) in parentheses.]

L.c. diagrams, shown in Figs. 5–7, give clear evidence that the phosphorylase now has to deal with primers so crowded that it is difficult for amylose chains to grow from all the potential sites. Data evaluated by curve analysis of the l.c. diagrams are summarized in Table IV.

The 3-functional primer gives rise to three components in the molecular-weight ratios of 3:2:1 (see Fig. 5). This means that products have also been formed with only one or two primer chains elongated. As may be seen from Table IV, the 2-star molecule is preferentially formed.

The 4-functional primer leads to a bimodal distribution with a molecular-weight ratio of 2:1 (see Fig. 6, and Table IV). The two components are present in about equal proportions. We were not yet sure whether the components carry four and two branches or only two and one. Compared with that of the higher-molecular-weight polymer, the peak of the lower-molelcular-weight polymer has a smaller width, and thus, a narrower chain-length distribution, than the former. This contrasts with the normal situation, in which the signals of components which are eluted at a later stage are broadened due to diffusion effects.

The 6-functional primer gives mainly two peaks, and a third, weaker and broader, one in the lower-molecular-weight region (see Fig. 7). The latter increases

TABLE IV

PHOSPHOROLYTIC SYNTHESES WITH 3-, 4-, AND 6-FUNCTIONAL PRIMERS. CHARACTERIZATION OF THE TRICARBANILATED POLYMERS BY 10-MPa L C -G P C AND VISCOSITY

Product	*Mg of primer/ 4 g of G-1-P*	*D.p. of components*			*Percent of components*			$\overline{D.p.}$ *(10 MPa l.c)*	$D.p._{\eta}$	D.p.$_{\eta}$ *after debranching*
		1	*2*	*3*	*1*	*2*	*3*			
S3*-1[a]	70	595	365	215	25	50	25	385	375	200
S3*-2	40	860	530	375	7	60	33	500	400	380
S3*-3	20	1925	805	685	3	60	37	775	760	n.d.[b]
S4*-1[c]	60	640	340	—	51	49	—	495	490	n.d.
S4*-2	30	990	570	—	44	56	—	800	920	530
S6*-1[d]	130	385	210	105	54	40	6	300	220	n d.
S6*-2	80	555	335	160	43	45	12	405	330	n.d.
S6*-3	20	(3390)[e]	1035	670	8	44	48	1040	975	660
S6*-4	10	(4850)[e]	1430	1010	7	46	47	1470	1700	n.d.

[a]S3*, primer coupled to 1,3,5-benzenetriacetic acid (see Experimental). [b]Not determined. [c]S4*, primer coupled to (ethylenedinitrilo)tetraacetic acid (see Experimental). [d]S6*, primer coupled to *S*-(aminoethyl)-perthio-D-glucitol (see Experimental). [e]Exclusion limit of the gel.

considerably with decreasing primer concentration, and the highest-molecular-weight peak decreases correspondingly. The molecular-weight ratios derived from curves a and b in Fig. 7 are ~3:2:1. A considerable broadening is observed at the lowest primer concentration (see curve d in Fig. 7).

Because l.c. diagrams do not allow an unequivocal assignment of the components to the number of amylose chains introduced, some of the samples were saponified by treatment with sodium methoxide, in order to split amide bonds linking the branches. Phenylcarbamate substituents are concomitantly split. The recarbanilated amyloses investigated by l.c. then give only a single component with a fairly narrow molecular-weight distribution. A tendency to tailing was observed in the case of the debranched 3- and 6-functional stars. The $d.p._{\eta}$ values listed in Table IV (last column) were found to be equal to, or slightly lower than, those of the lowest-molecular-weight component. From these results, it may be concluded that the mixture of products which have been formed from the 3- and 6-functional primers consists of three-, two-, and one-arm molecules, and that, from the 4-functional primer, most probably two- and one-arm molecules are formed. Taking into account that some degradation may have occurred on saponification, amylose chains of a rather uniform length must have been grown.

Data from light-scattering and viscosity measurements are listed in Table III. As might be expected, an influence of the branched character cannot be detected. Due to the small number of chains, Zimm plots do not show the upturn curvature of the particle-scattering factor typical of star molecules with long, homodisperse chains[16–18]. The molecular-weight dependences of the intrinsic viscosity (see Fig. 8) and the radius of gyration of the star polymers (see Fig. 9) show good agreement with those of the amylose tricarbanilates, marked by the solid lines in these Figures.

Comb polymers. — Preliminary studies have been carried out with multi-

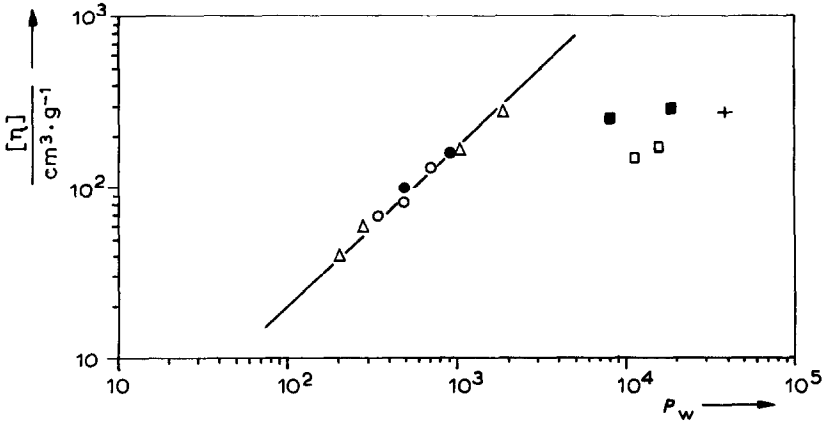

Fig 8 Dependence of the intrinsic viscosity ([η]) on degree of polymerization (P_w) for star and comb polymers. [Key: Star polymers; (○) S3*, (●) S4*, (△) S6*, and comb polymers derived from poly(vinylamine) with d s . (□) 0 1, (■) 0 3, and (+) 0 6 Solid line, amylose tricarbanilates[8].]

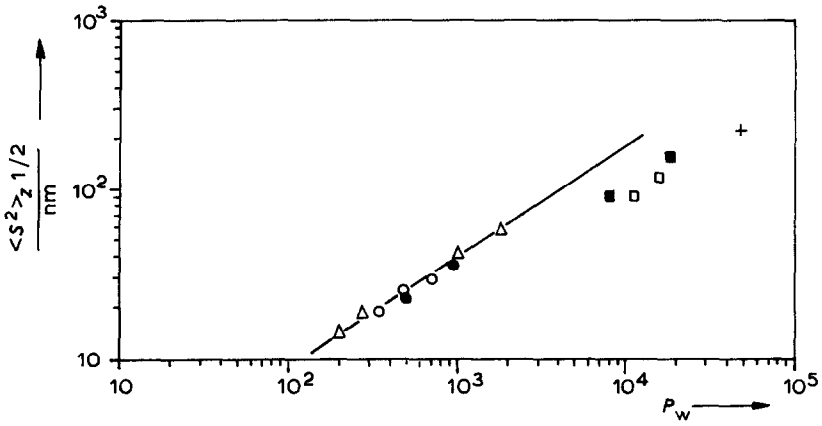

Fig 9 Dependence of the radius of gyration ($\langle S^2 \rangle_z^{1/2}$) on degree of polymerization (P_w) for star and comb polymers [For symbols, see Fig. 8) Solid line, amylose tricarbanilates[10,11]]

functional primers derived from poly(vinylamine). Branch density varied from d.s. = 0.1 to 0.3 to 0.6, *i.e.*, the polymer backbone of $\overline{\text{d.p.}}$ 450 contained about 45, 150, and 300 malto-oligosaccharide primers. Due to their free charges, the partially substituted poly(vinylamine)s interact with charged groups of the protein. In fact, a slight precipitate was observed on incubation of poly(vinylamine) with the phosphorylase solution. We therefore blocked the remaining amino groups of the backbone chain by reaction with D-gluconolactone, which has no primer activity.

Phosphorolytic syntheses were performed with relatively low primer concentrations, to prevent the formation of shorter amylose chains in the poorly soluble range[19] of d.p. 80, which at the high, local concentration would give rise to premature precipitation of the compounds. Nevertheless, synthesis proceeded at a very high rate. This could be taken as an indication that most of the primer endgroups are freely accessible to the potato phosphorylase.

From the high molecular weights measured by light-scattering (see Table III), and from the molecular-weight dependences of the intrinsic viscosity (see Fig. 8) and the radius of gyration (see Fig. 9), the highly branched structure of these comb polymers is clearly seen. Zimm plots gave a characteristic upturn of the particle-scattering factor at higher angles. Obviously, steric hindrance is much smaller than in the star-shaped primers. This may result from a greater flexibility of the backbone chain and the lower density of the branch points as compared with the rather stiff and densely packed arrangement in the oligofunctional carriers.

DISCUSSION

In our studies with di-, oligo-, and multi-functional primers, we wished to know whether phosphorolytic synthesis would proceed in the same way as with unbound malto-oligomeric primers, leading to amylose chains of a uniform length.

If it does, this would allow the production of macromolecules of different shape carrying amylose chains of any desired length. With regard to previous studies[16,17] and theoretical considerations[18,20], special interest was focused on regularly shaped, star polymers. Unfortunately, we have not been successful in preparing star molecules of this type, due to restrictions of the enzyme. Therefore, in the following discussion, we shall concentrate mainly on possible reasons that may explain our findings.

It has been known for a long time that animal phosphorylases have a considerably higher affinity for branched than for linear substrates[21,22]. Potato phosphorylase is reported not to distinguish between branched and unbranched substrates[23,24]. Remarkably, glycogen is a poorer substrate than amylopectin in spite of the basic similarity of their branched structures. We found that potato phosphorylase utilizes only 15–20 end-groups (2%) of the more densely branched glycogen, whereas a greater number, possibly all, of the 10,000 end-groups are accessible in the less densely branched amylopectin[16,25]. When carrying out the same experiments with muscle phosphorylase, 400–450 (50%) of the glycogen end-groups could be elongated[17]. It still remained an open question whether the differences between the affinities of the two enzymes are related mainly to topological details of the branched structures, *e.g.*, density of the branch points, or to the length of the outer chains as well.

Phosphorylase from animal sources, a key enzyme in glycogen metabolism, has been extensively studied over many years. Depending on concentration, temperature, and ionic strength, the enzyme exists in tetrameric and dimeric forms with molecular weights of 4×10^5 and 2×10^5 g/mol, respectively. The active form is the dimeric species when phosphorylated on serine 14. Much progress was made on the question of substrate-binding specificity when the complete, three-dimensional structure was resolved by X-ray crystallography[26–30]. The two active sites of the dimer are located at the subunit interface, sharing residues from both[28]. This explains why the monomers, although carrying binding sites for α-D-glucosyl phosphate and orthophosphate adjacent to the catalytically involved pyridoxal 5′-phosphate group, are obviously inactive[28,31]. X-Ray crystallographic studies revealed a separate substrate-binding site, the so-called "glycogen storage site", 25 Å distant from the active center. When crystals are soaked with maltopentaose or other linear or branched malto-oligosaccharides, binding is found to occur at this storage site, and not at the active site[29]. This is thought to reflect a dissociation constant at the active site at least 20-fold greater than at the storage site. Previous occupation of the storage site seems to be responsible for the dissociation of the tetrameric into the active dimeric form[29].

From recent amino acid sequence comparison, it was shown that potato and muscle phosphorylase are similar in terms of primary structure, and also in the active site with the bound cofactor[32]. However, these two enzymes are rather different from each other in their secondary structures. Results from affinity gel-electrophoresis indicate that, in the potato phosphorylase, there is no separate

storage-site in addition to the active site[33]. As demonstrated by competitive inhibition, the enzyme showed equal affinity for amylopectin and maltopentaose, but again a higher affinity for amylopectin than for glycogen[33]. The potato phosphorylase has two subunits, suggesting two active sites per molecule. Partial dissociation into monomers was observed at protein concentrations[34] below 0.5 mg/mL. These monomers are also assumed to be active, but this point has not been fully clarified.

We have included the foregoing discussion in order to facilitate interpretation of the results with regard to the primer concentration. It should be noted that each series of syntheses using different amounts of primer has been run in parallel under equal conditions, *i.e.*, with the same sample of primer and equal amounts of G-1-P and phosphorylase. Digests sometimes differed only in their degree of dilution. In order to prevent precipitation of shorter, less-soluble amylose chains which will be synthesized at a high primer concentration, a higher dilution of the digests was necessary than in the case of a lower primer concentration[16].

In each series, an increasing chain-length was observed with decreasing primer concentration, as anticipated. Syntheses with difunctional primers were found to proceed in the expected way. The results confirm that both primer ends are equally accessible to the potato phosphorylase, and grow simultaneously to a molecular-weight distribution as narrow as with monofunctional primers. No differences exist between samples with B-blocks $-(CH_2)_6-$ and $-(CH_2)_{12}-$, and when $-(CH_2)_n-$ is replaced by $-(CH_2CH_2O-)_6-$. This finding is in good agreement with previous, kinetic measurements[9]. The observation of a broader molecular-weight distribution of A–B–A polymers prepared from the primer containing a longer poly(oxyethylene) block of d.p. ~45 has to be attributed to nonspecific binding by interaction of the polar groups of the poly(oxyethylene) with parts of the phosphorylase polypeptide chain more remote from the active center of the enzyme Consequently, dissociation of the enzyme–substrate complex and free diffusion of the reactants will not occur as readily as required to meet the conditions enumerated in the Introduction.

The results obtained with the oligofunctional primers are closely connected with the large differences in specificity of the potato phosphorylase for amylopectin and glycogen. They show that differences are not based on the length of the outer chains but on details of the branched structure.

The most interesting finding is the formation of only two components in nearly equal amounts from the 4-functional primer, independent of the primer concentration, *i.e.*, with variable ratio of primer end-groups to monomer and to phosphorylase molecules, respectively. In order to explain the preference of muscle phosphorylase for multiply branched substrates Hu and Gold[22] proposed a model in which two chain termini from the same branched molecule are simultaneously bound to the phosphorylase dimer. Although potato phosphorylase has no preference for branched substrates, the Hu and Gold model seems to work as well. In the carrier, (ethylenedinitrilo)tetraacetic acid, carboxyl groups are arranged in two identical pairs. The average distance between members of a given pair is much

smaller than the average distance between members of two different pairs. Depending on the distance of separation, cooperation of two binding sites for each pair of linked primers would offer a reasonable explanation for the formation of only two compounds and no three-arm molecules. Whether pairwise binding really leads to a simultaneous growth of both primer ends cannot as yet be answered by kinetic measurements. Debranching experiments, however, favor a mixture of two- and one-arm molecules, rather than a mixture of four- and two-arm molecules.

Pairwise binding could also explain the results with the 3-functional primer. One of the three components, the two-arm molecule, is preferentially formed, independent of the primer concentration. Apparently, the attack of all primer ends at once has a better chance at a high primer concentration (see Table IV), and this could be explained by the fact that a higher primer concentration would help to overcome sterically unfavorable alignments. It appears that the ratio of the components, shifting to a smaller number of branches with decreasing primer concentration, is largely determined by the initiation step. One can imagine that one primer chain has entered the first active site to its full length, and that a second cannot then enter the adjacent site completely, due to steric constraints. This would then lead to a much lower initiation rate, comparable perhaps to that of maltotriose. However, as soon as the short primer chains have been elongated to a certain length and a greater flexibility, steric hindrance would play a minor role. Chain growth can then proceed quite regularly. This would explain both the relatively uniform length of the amylose chains in all components of different numbers of branches and the tendency of tailing towards shorter chains observed with the 3-functional primer.

The same considerations can be applied to the results with the 6-functional primer Pairwise attack is indicated, as three components are clearly visible in the l.c. curves, at least at a higher primer concentration. The middle component, most probably the two-arm molecule, is preferentially formed, and its relative amount is independent of the primer concentration.

Our approach to investigating the mechanism of enzymic growth by a detailed polymer structure analysis, *i.e.*, the combined application of light-scattering, viscosity, and 10-MPa l.c., proves to be a valuable supplement to information from kinetic measurements. According to the present studies with di- and oligo-functional primers, it is mainly the difference in the density and topology of the branch points in amylopectin and glycogen which renders them different in their accessibility to the potato phosphorylase. Preliminary observations with the multifunctional, comb-shaped primers support this conclusion. Because pairwise binding of primer end-groups from the same molecule is also indicated by our studies, this model does not fully explain the preference of muscle phosphorylase for highly branched substrates. Thus, the specificity of muscle phosphorylase for glycogen has to be attributed to a special role of the storage site which does not exist in potato phosphorylase.

EXPERIMENTAL

Preparation of malto-oligomeric primers. — Pure malto-oligomers in the range of maltopentaose to maltooctaose were obtained from an alpha-amylolytic digest of amylose by g.p.c. on Bio-Gel P-2 or P-4, using a large-sized column[35] (5 × 150 cm). The preparation of aldono-1,5-lactones was effected by electrolytic oxidation[35]. Terminal amino groups were introduced by the reaction of the aldonolactone with a two-fold molar excess of ethylenediamine in dimethyl sulfoxide at ambient temperature[35]. The malto-oligosaccharide aldonolactones and the *N*-(2-aminoethyl)aldonamide derivatives were purified by ion-exchange chromatography and, more effectively, by chromatography on Bio-Gel P-4 containing a small number of carboxyl groups introduced by saponification[1] (see Chromatographic methods).

Primers were bound to different carriers by amide linkages. Difunctional primers were obtained by coupling of the aldonolactones to aliphatic diamines, H_2N–$(CH_2)_n$–NH_2 (n = 2, 6, and 12) and to α,ω-diamino-substituted hexa(oxyethylene) and poly(oxyethylene) (mol. wt. 2000)[7] in ethylene glycol[1,6,36] at 70–80°. The 3- and 4-functional primers were prepared by coupling of the *N*-(2-aminoethyl)aldonamide derivatives to the fully substituted *p*-nitrophenyl esters of 1,3,5-benzenetriacetic acid and of (ethylenedinitrilo)tetraacetic acid, respectively, in dimethyl sulfoxide[1,4] at 40°. The 6-functional primer was prepared by coupling of maltoheptaonolactone to *S*-(aminoethyl)-perthio-D-glucitol[7] in ethylene glycol[6] at 70°. The samples were carefully purified by chromatography on a column of modified Bio-Gel P-4 or P-10, taking advantage of both the molecular-sieve effect of the gel and its ion-exchange properties[1] (see Chromatographic methods).

Poly(vinylamine), used as a carrier for multifunctional primers, was prepared from vinyl isocyanate (BASF) converted into *N*-vinyl-*tert*-butylcarbamate followed by radical polymerization[5,37]. Molecular weights of different preparations were in the range $\overline{d.p.}$ 420–480 (vapor-phase osmometry). A suitable method for coupling under homogeneous conditions was found in the reaction of the poly(vinylamine hydrochloride) with malto-oligosaccharide aldonolactones in ethylene glycol at 70° in the presence of triethylamine[5]. Different degrees of substitution (d.s.) were obtained by varying the molar ratio of aldonolactone to amino groups. Subsequent substitution of the remaining amino groups by reaction with D-gluconolactone was performed accordingly.

Purification of potato phosphorylase. — In the first step, the pH of the juice from 5 kg of potatoes was adjusted to 7 by addition of conc. ammonia, and the mixture was kept for 45 min at 55.5°. Heat treatment resulted in complete deactivation of the contaminating alpha amylase. Solid ammonium sulfate (100 g/L) was then added to a density of 1.08, and the precipitate formed was separated by centrifugation for 30 min at 5000 r.p.m. To the clear supernatant liquor was added ammonium sulfate (250 g/L) to a density of 1.150. The precipitate containing phosphorylase was separated by centrifugation, and then suspended in 0 05M 2,2′,2″-nitrilotriethanol-HCl buffer, pH 7.0 (75 mL). After standing overnight, the turbid

solution was clarified by centrifugation in a Beckman ultracentrifuge for 1 h at 15,000 r.p.m. The solution, having a specific activity of 0.24 unit/mg of protein, as compared to 0.048 unit/mg of protein in the potato juice[38], retains its activity for months when stored at 4°.

Phosphorolytic synthesis. — For a series of syntheses, stock solutions were prepared. (*i*) D-Glucosyl phosphate, disodium salt (Boehringer, Ingelheim), was dissolved in water, adjusted to pH 6.2 by addition of 2M acetic acid, and diluted to a concentration of 1 g/10 mL. (*ii*) Primers were dissolved in water (2 mg/mL). Typical digests contained in a total volume of 80 mL were: 4 g (40 mL) of G-1-P, M sodium citrate buffer, pH 6.2 (8 mL), primer (*e.g.*, 2.0 to 110 mg), and phosphorylase solution (4 mL). Phosphorylase was added last, under stirring. Digests were usually incubated at 37°. In the case of primer concentrations, leading to the formation of amyloses in the range of d.p. 50 to 250, which are known to be rather unstable in aqueous solution[19], the digests were diluted to 160 mL or 320 mL and incubated at 45°. The reaction was monitored by photometric determination of the liberated phosphate, and terminated by heating in a boiling-water bath as soon as equilibrium had been reached (75–80% of conversion of G-1-P). The precipitated protein was removed by filtration through a fluted filter-paper, and 10% (v/v) 1-butanol was added at 80° to the clear, aqueous solution. As described elsewhere[16,39], the butanol complex was reprecipitated twice, and the products isolated[40] by treatment with methanol and, finally, with ether; yield: 1.3 g.

Preparation of the tricarbanilates. — The tricarbanilyl derivatives were prepared by the reaction[16] of the products with phenyl isocyanate in pyridine at 80°.

Debranching of the producs. — Sodium methoxide was freshly prepared by dissolving sodium (6 g) in abs. methanol (100 mL). The tricarbanilated sample (1 g) was dissolved in 1,4-dioxane (15 mL), and sodium methoxide (6 mL) was added. The precipitate formed after heating for 1 h at 80° was filtered off through a glass funnel, and thoroughly washed with methanol and, finally, with acetone. Purification was carried out by reprecipitation from dimethyl sulfoxide in methanol. After saponification, all products gave a deep-blue color with iodine.

Chromatographic methods

Ion-exchange chromatography. — Chromatographic separations were carried out by using the strong cation-exchange resin Amberlite IR-120 (Serva), and a strong anion-exchange resin, type III (Merck).

Gel-permeation chromatography (g.p.c.). — Separations were performed on columns (with dimensions 5 × 150 cm and 1.6 × 90 cm) of Bio-Gel P-2, P-4, and P-10 (−400 mesh) (Bio-Rad Laboratories) and by elution with water at 50° under hydrostatic pressure. Cation-exchange properties were obtained by contact of the fresh gel with aldonic acids or with ethylenediamine, both leading to a partial hydrolysis of acrylamide bonds (modified Bio-Gel). More details for the purification of the various samples by g.p.c. on fresh and modified Bio-Gel are given elsewhere[1,6,35].

10-MPa Liquid chromatography (l.c.). — A liquid chromatograph equipped with a differential refractometer and a variable-wavelength detector was used (Fa. Knauer, Berlin). U.v. absorption was recorded at the maximum absorption of amylose tricarbanilates at 236 nm. The chromatograph was fitted with the g.p.c. set (Polymer Laboratories, U.K.) consisting of three columns of cross-linked poly(styrene) (7.7 × 300 mm) with pore sizes of 10^3, 10^4, 10^5 Å (set 6), and an additional column with a pore size of 10^6 Å (set 6 1). The mobile phase was degassed tetrahydrofuran (THF) (Lichrosolv®, Merck). From sample solutions in THF containing 20 mg/mL, 20 μL was injected and run at a flow rate of 0.98–0.99 mL/min at a pressure of 10–11 MPa (set 6) and 1.00–1 01 mL/min at a pressure of 16–17 MPa (set 6.1). The calibration of the elution volumes was carried out with 9 poly(styrene) standards (Pressure Chemical Co., Pittsburgh) in the molecular-weight range of 0.08–120 × 10^4 mol and 18 samples of tricarbanilates of synthetic amyloses in the range of 1.8–300 × 10^4 g/mol. As may be seen from the calibration curves in Fig. 10, at equal molecular weights, amylose tricarbanilates are eluted at somewhat higher V_e values than poly(styrene)s, indicating differences in the hydrodynamic volumes of the two polymers.

Physical measurements

Viscosity measurements. — Intrinsic viscosities of the tricarbanilated products were determined in 1,4-dioxane at 20° with an Ostwald viscosimeter, and, in the high-molecular-weight range (mol. wt $>10^6$), with an Ubbelohde viscosimeter. Dependence of the intrinsic viscosity on shear rates was observed for high-molecular-weight A–B–A samples, but not for comb-shaped polymers (see Table III)

Light-scattering measurements. — Static light-scattering measurements were made with a Sofica light-scattering photometer (Fica) equipped with a mercury-vapor lamp and a vertically polarizing filter Rayleigh scattering was recorded over

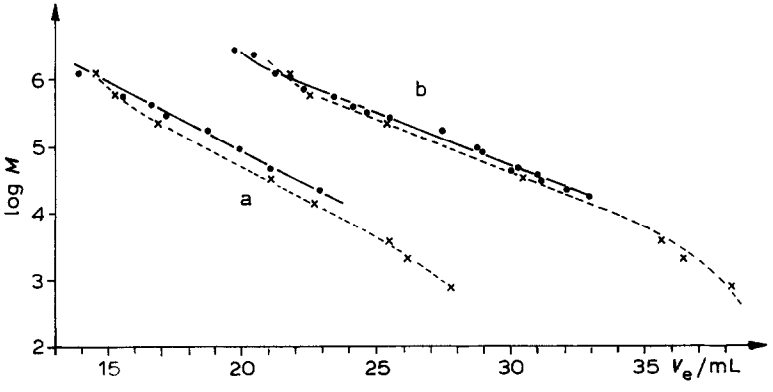

Fig 10. Calibration of elution volumes from 10-MPa l c –g p.c on microgel set 6 (a) and set 6 1 (b), with homodisperse amylose tricarbanilates (——) and poly(styrene) standards (----) (THF, 20°)

the angular range of 20–150° in steps of 5° at 436-nm wavelength at 20°. The samples were dissolved in 1,4-dioxane. Stock solutions containing 2–10 g/L were diluted to four different concentrations, ranging from 0.04–1.6 g/L for the highest molecular weight, and 2–8 g/L for the lowest molecular weight. The solutions were clarified by centrifugation for 1 h at 20,000 r.p.m. in a Beckman ultracentrifuge L50 with a swinging-bucket rotor, model SW 25.1. The reduced scattering intensity kc/R_θ was plotted (according to Zimm) against $q^2 + kc$ ($q^2 = (4\pi/\lambda)^2 \sin^2 \theta/2$, with $\lambda = (\lambda_0/n)$. The refractive index of the solvent 1,4-dioxane is $n = 1.433$ at 20°. The refractive index increment of amylose tricarbanilate $dn/dc = 0.1627$ mL.g^{-1} at^{10,40} 436 nm.

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