CROSSLINKING STUDIES WITH DIFFERENT LENGTH DITHIOBISALKYLIMIDATES

(I) SOLUBILIZED ERYTHROCYTE SPECTRIN

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SUMMARY: A series of different length dithiobisalkylimidate homologues was synthesized *via* Bunte salts from corresponding ω-chloronitriles. And by using these reagents, crosslinking of human erythrocyte spectrin was examined. Shorter reagents formed three bands in the region expected for a dimer, all of which were identified as the bands 1-2 associate: bands 1 and 2 polypeptides were crosslinked only each to one another and not to itself. Longer reagents, however, produced higher multimers, which was enhanced by higher temperatures and divalent cations, and depressed by Triton X-100 and deoxycholate. The results suggest the validity of the chain length of the reagents in discriminating between dimer and multimer of spectrin.

INTRODUCTION

Molecular architecture of erythrocyte spectrin *in situ* is one of most interesting subjects in membrane biochemistry. Spectrin-like proteins are now reported in many other eukaryotic cells and are supposed to construct cytoskeleton in the cytoplasmic face of surface membranes. They are not only a basis for cell shape and movement, but also control the distribution of membrane components. These proteins are also supposed to play a crucial role in mediating the external stimuli accepted by membrane components exposed on the outer surface into cytoplasmic processes or *vice versa* (1).

In the present paper, we report the syntheses of cleavable bisimidates, dithiobisalkylimidates, having different distances between two functional groups. And by using these different length reagents, crosslinking of spectrin was examined with an aim to investigate the molecular architecture of spectrin in erythrocyte membrane. The results of crosslinking of membrane spectrin will soon be published elsewhere.

Abbreviations used: DTBP, dithiobispropionimidate; DTBB, dithiobisbutyroimidate ; DTBV, dithiobisvaleroimidate; DTBE, dithiobisenanthoimidate; DTBU, dithiobisundecanoimidate; SDS, sodium dodecyl sulfate; NEM, N-ethylmaleimide; Cu-P, cupric sulfate-phenanthroline 1:2 molar mixture; DMS, dimethyl suberimidate.

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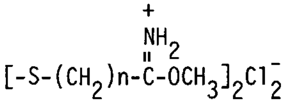
MATERIALS AND METHODS

Syntheses of dithiobisalkylimidates. ω-Chloronitriles were obtained from corresponding diols by usual methods (2-5). 3-Chloropropionitrile was obtained commercially and used after distillation. Dicyanodisulfides were synthesized via Bunte salts from respective ω-chloronitriles by a modification of the method employed for dithiobisbutyronitrile by Stoner and Dougherty (6) as follows: 0.1 mole of ω-chloronitrile was dissolved in alcohol and 0.1 mole of aqueous sodium thiosulfate was added. The mixture was refluxed on a steam bath with stirring for 2-48 h. The reaction was so slow in higher homologues that prolonged refluxing was necessary. After the end of the reaction, 0.1 atom of iodine was added while hot and the mixture was cooled to room temperature with stirring. For higher homologues, the mixture was further refluxed for 20-60 min after the addition of iodine. After cooling, excess iodine was reduced by sodium thiosulfate untill brown color dissipated. The mixture was neutralized to pH 7 and extracted with chloroform three times. After washing with water, drying over anhydrous magnesium sulfate and evaporation of the solvent, obtained dicyanodisulfides were purified by passing through a silica gel column. Chloroform-ether 2:1 mixture was used as the eluant for lower homologues and ether for higher homologues. The products moved as translucent zone and could be collected easily in one fraction.

Dicyanodisulfides thus obtained were then converted to dithiobisimidates by the method of McElvain and Schroeder (7). Corresponding free bases were also obtained by the reaction of dithiobisimidates with triethylamine in chloroform. All the free bases obtained showed no resonances other than indicated in the table below in their NMR spectra and no evidence for nitrile or amide II band in their IR spectra.



$$[\text{-S-}(CH_2)n\text{-}\overset{\overset{+}{NH_2}}{\overset{\|}{C}}\text{-}OCH_3]_2Cl_2^-$$

	n	Yield*	Elemental analysis												DP**
			Calcd.						Found						
			C	H	N	O	S	Cl	C	H	N	O	S	Cl	
DTBP	2	68	31.07	5.87	9.06	10.35	20.73	22.93	31.30	5.89	9.00	10.06	20.63	23.12	123-5
DTBB	3	68	35.61	6.58	8.30	9.49	19.01	21.02	35.87	6.73	8.05	9.51	18.87	20.97	107-9
DTBV	4	70	39.45	7.17	7.67	8.76	17.55	19.40	39.42	7.06	7.89	8.65	17.80	19.18	98-100
DTBE	6	59	45.60	8.13	6.65	7.59	15.21	16.82	45.64	8.07	6.72	7.73	15.34	16.50	92-3
DTBU	10	54	54.01	9.44	5.25	6.00	12.10	13.28	54.22	9.50	5.13	6.12	11.88	13.15	92-4

* based on ω-chloronitrile (%)
** decomposition point (°C)

$$[\text{-S-}(CH_2)n\text{-}C(=NH)OCH_3]_2$$

	n	IR(cm^{-1})		NMR (ppm)				
		N-H	C=N	$^{N}_{O}{>}C\text{-}CH_2\text{-}$	-(CH_2)n-	-CH_2-S	CH_3O	=NH
DTBP	2	3280	1650		2.77 (m, 4H)		3.70(s,3H)	6.72(b,1H)
DTBB	3	3280	1650	2.38(t,2H)	2.00 (q, 2H)	2.68(t,2H)	3.70(s,3H)	6.64(b,1H)
DTBV	4	3280	1650	2.28(t,2H)	1.72 (m, 4H)	2.69(t,2H)	3.70(s,3H)	6.5 (b,1H)
DTBE	6	3280	1650	2.24(t,2H)	1.2-1.8(m, 8H)	2.67(t,2H)	3.70(s,3H)	6.5 (b,1H)
DTBU	10	3310	1650	2.23(t,2H)	1.2-1.8(m,16H)	2.68(t,2H)	3.70(s,3H)	

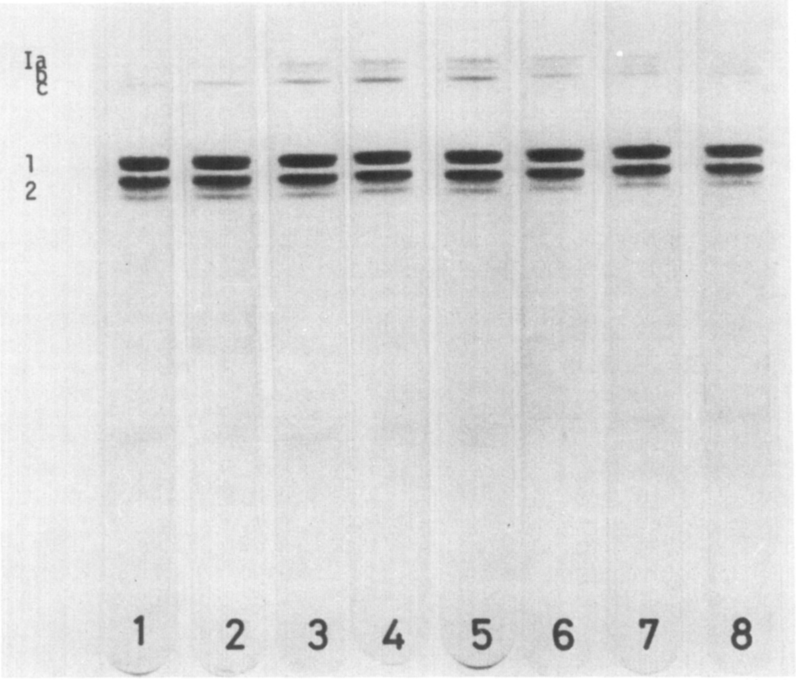


Fig. 1. Effect of concentration of DTBP (n=2) on crosslinking of spectrin. Samples 1 to 8 were crosslinked at 25 °C for 60 min by 0, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 mM of DTBP, respectively.

Spectrin. Erythrocyte spectrin was prepared from freshly drawn human blood (A, Rh^+) according to Nicolson and Painter (8), stored at 4 °C and used within a few days. Prolonged incubation, especially at higher temperatures, resulted in spontaneous crosslinking of their polypeptides due to disulfide formation. And NEM-treatment of spectrin did not have any appreciable effects on crosslinking by dithiobisimidates. Hence NEM-treated spectrin was supplied for crosslinking except that by Cu-P. NEM-treated spectrin was prepared by incubating spectrin with 1.5 mM NEM for 30 min at 25 °C in 10 mM Tris-HCl [pH 8.0 (10T(8))], followed by dialysis against the same buffer at 4 °C overnight.

SDS polyacrylamide gel electrophoresis. The procedure of Fairbanks et al (9) was essentially followed. The gels were cast of 3.2 % acrylamide and the concentration of Bis was 3.7 % of the acrylamide monomer.

Crosslinking. Usually about 0.4 mg/ml of spectrin was crosslinked by reagents freshly dissolved in 10T(8) before use. The concentrations of reagents and additives, reaction times and temperatures actually used are noted in the figure legends. The reaction was stopped and the sample dissolved for electrophoresis by adding fivefold SDS concentrate and by warming at 35 °C for 30 min. Since no crosslinking occurred in the presence of SDS, dissolving spectrin in SDS is considered to be adequate procedure for stopping the reaction (10).

Miscellaneous. Protein was determined by the method of Lowry. The values for the molecular weights of all the major polypeptide bands of erythrocyte ghost were used as standards of molecular weight calibration. Gels were stained with coomassie brilliant blue and densitometered on Cosmo Densitometer, Super Click, Model D-101. Infrared and NMR spectra were measured with JASCO IRA-2 Grating Infrared Spectrophotometer and Varian XL-100 NMR Spectrometer.

RESULTS AND DISCUSSIONS

Fig. 1 illustrates the effect of increasing concentrations of DTBP (n=2) on spectrin in 10T(8) at 25 °C. Three bands were observed in the region of molecular weight about 400-460 K daltons, each of which was designated as Ia, Ib and Ic from the top of gels. The formation of these three bands was reached near maximum at about 2 mM of the reagent concentration. At higher concentrations, their amount decreased progressively without apparent formation of higher products. At higher reagent-to-protein ratio, monofunctional reaction of the bifunctional reagent may exceed over bifunctional one. This explains the observed concentration dependence of crosslinking by DTBP. The mobility of the crosslinked products was consistent with that expected for a dimer. And the formation of multiple bands in this region was also reported by using other reagents (10, 11). The interpretation of the results, however, has not been in an agreement. The point in the conflicting arguments is the question as to whether bands 1 and 2 polypeptides are crosslinked each to itself as well as to one another (12) or they are crosslinked only each to one another and not to itself (13). The results of cleaving experiments by Hulla and Gratzer (14) and by Steck(10) are not so distinct. DTBP yielded bands Ia, Ib and Ic in different ratio, but resulted in an equal shift of bands 1 and 2 into these forms. Cu-P also yielded three bands in the positions identical with Ia, Ib and Ic. Ic was predominant at lower reaction conditions (for example, 0.01 mM Cu-P at 25 °C for 10 min), but was converted to Ia at higher conditions. And relative ratio between residual bands 1 and 2 was nearly unchanged under these conditions. Glutaraldehyde and formaldehyde produced only one band in the position identical with Ic, while bands 1 and 2 decreased in an equal extent. These results suggest that all of the multiple bands in the dimer region are the heterodimer

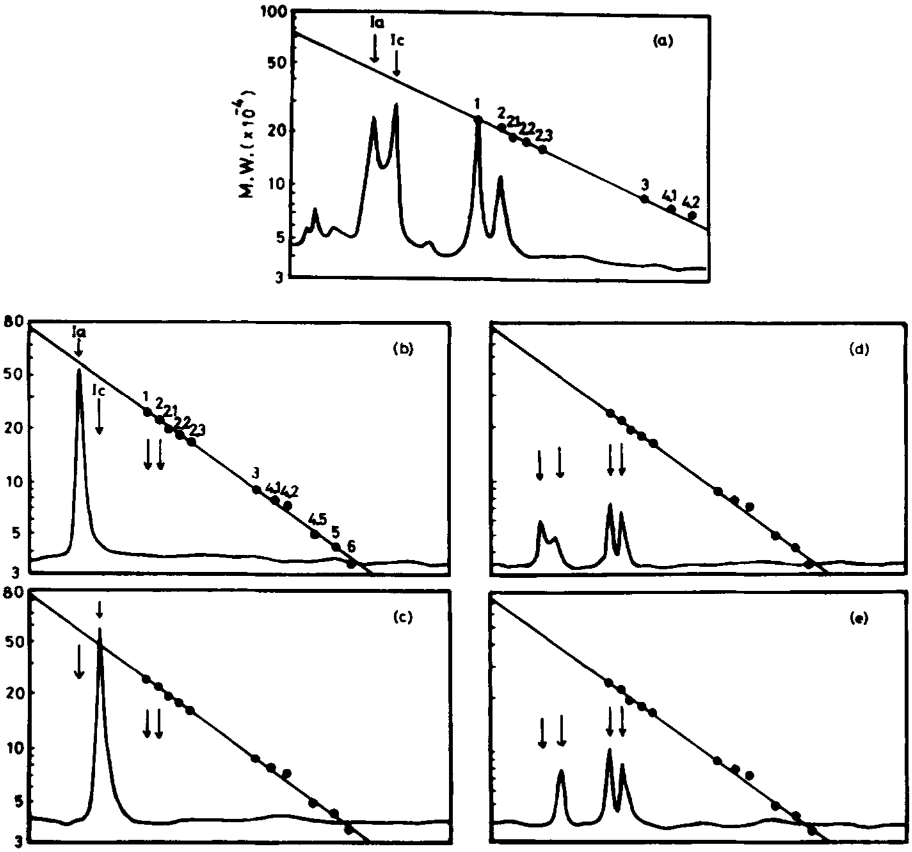


Fig. 2. Cleavage of crosslinks. Spectrin was crosslinked by 2 mM of DTBP for 60 min at 40 °C. Samples were dissolved in SDS concentrate lacking β-mercaptoethanol and electrophoresed for 3 h at 8 mA/gel. One gel was stained (a). The zone corresponding to Ia-c was cut out of remaining unstained gels into small pieces of about 2 mm width sequentially. Gel slices were treated with the standard detergent concentrate lacking (b, c) or containing (d, e) 10 % β-mercaptoethanol at 35 °C overnight. The slices were then loaded on to fresh gels and electrophoresed for 1 h and 30 min. The gels were stained and scanned.

between bands 1 and 2 polypeptides. In order to confirm the above assumption, we tried cleaving experiment. The crosslinked products by dithiobisimidates could be cleaved easily under mild conditions owing to the introduction of disulfide bound (15). And the result of Fig. 2 clearly shows that both bands Ia and Ic yields bands 1 and 2 in nearly equal amount upon cleavage. The same result was also obtained for bands Ia and Ic formed by Cu-P. The difference in the number or site of crosslinking may result in changes in shape of products,

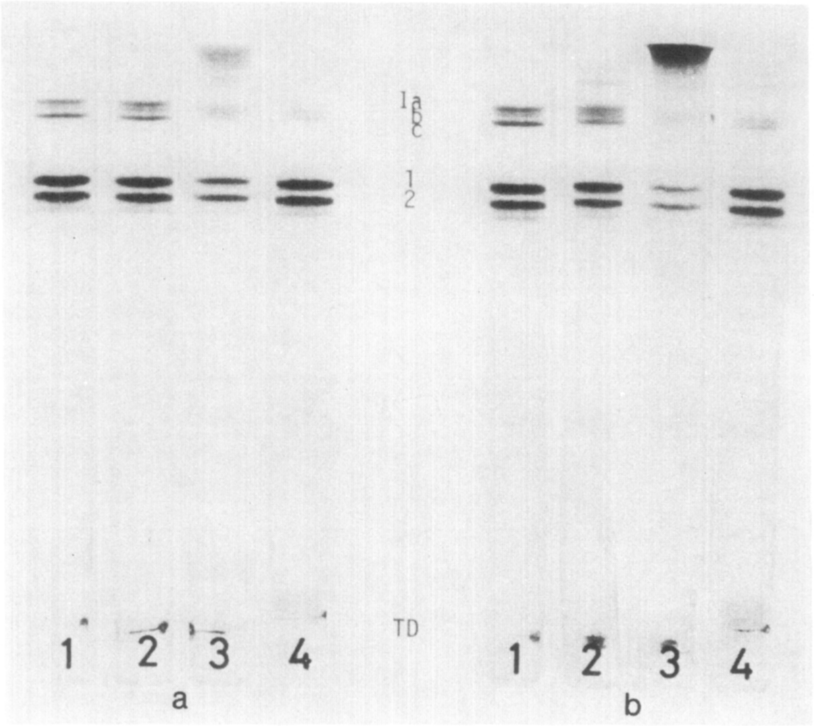


Fig. 3. Effects of chain length and temperature on crosslinking. Spectrin was crosslinked (a) at 25 °C for 2 h and (b) at 35 °C for 40 min by 2 mM of the following reagents: (1) DTBP (n=2), (2) DTBV (n=4), (3) DTBE (n=6) and (4) DTBU (n=10).

especially for rodshaped proteins like spectrin, and consequently in mobility of products during electrophoresis. The possibility that bands 1 and 2 may be a composite of several polypeptide species of a similar electrophoretic mobility (16), might be also in mind. Even if this is the case, the obtained results indicate that crosslink occurs only between polypeptide species belonging to different bands and not between those belonging to the same band.

In Fig. 3 the effect of the chain length of dithiobisimidates on crosslinking of spectrin is shown. The maximum distance between fixed amino groups for the reaction with the reagents is about 11.7 Å in DTBP, 14.2 Å in DTBB, 16.7 Å in DTBV, 21.7 Å in DTBE and 31.8 Å in DTBU. As illustrated, higher multimers beyond dimer was not formed by using shorter reagents, but their preferential

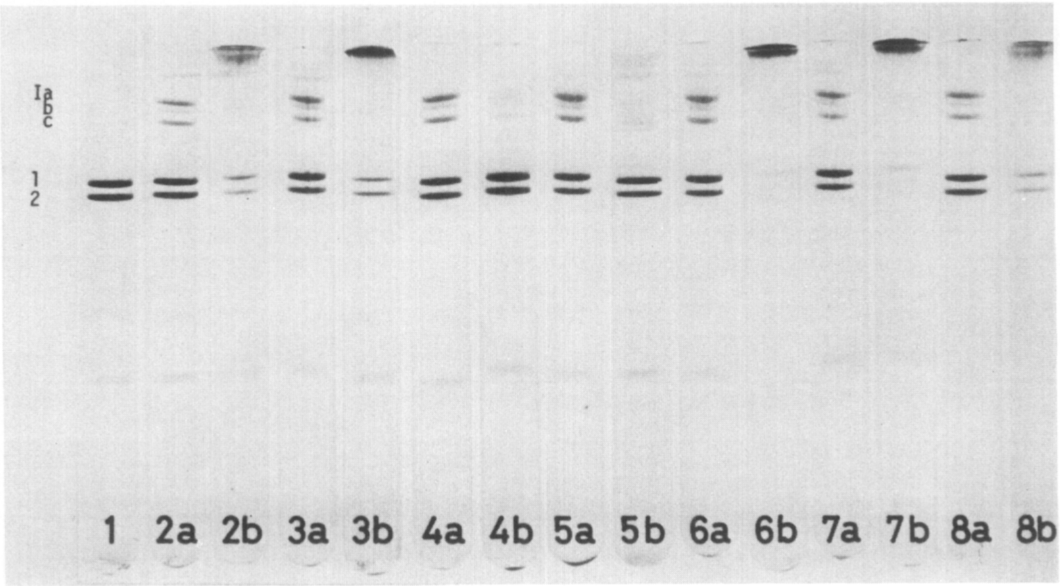


Fig. 4. Effects of detergents and divalent cations on crosslinking by DTBP (n=2) and DTBE (n=6). Sample (1) was incubated at 25 °C for 2 h without reagents. Samples (2) and (3) were treated at 25 °C for 2 h and at 35 °C for 40 min with 2 mM of (a) DTBP and (b) DTBE. Samples (4) to (8) were crosslinked at 25 °C for 2 h under the presence of the following additives: 0.5 % Triton X-100, 1 mM deoxycholate, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM EDTA.

formation occurred by longer reagents. And higher reaction temperatures enhanced the formation of these multimers by longer reagents. The amount of dimers formed by the reaction at 25 °C for 120 min was nearly identical with that at 35 °C for 40 min, when spectrin was crosslinked by DTBP. But under these conditions, the amount of multimers formed by DTBE was more pronounced at 35 °C. DTBU dissolved only partially at the reagent concentration used and the extent of the reaction was low.

Fig. 4 represents effects of detergents and divalent cations on crosslinking by DTBP and DTBE. SDS completely inhibited the crosslinking to both dimers and multimers, while Triton X-100 and deoxycholate selectively inhibited the formation of multimers by DTBE and did not that of dimers by DTBP. And no obvious dimer-bands were formed by DTBE in the presence of these detergents. Recently Schechter _et al_ (16) reported that deoxycholate dissociated multimers into dimer. On the other hand, divalent cations are known to aggregate spectrin into fibrillar structures in solution (17). And as expected, Ca^{++} and Mg^{++} promoted the formation of multimers by DTBE without having any effects on electrophoretic pattern of spectrin by themselves. Even in the presence of divalent cations, there was no appreciable changes in dimer-formation by DTBP.

To ascertain whether the observed crosslinking leading to higher multimers is intramolecular crosslinking or intermolecular one, we investigated the effect of the protein concentration on crosslinking by DTBE in the absence and presence of Ca^{++}. In both cases, multimers were formed up to 50-fold dilution of the protein concentration (8 μg/ml). And hence the crosslinking leading to higher multimers are considered to be those that are taking place between different subunits of the same associate.

Clarke (11) reported that spectrin might exist as dimer from the study of sedimentation properties and the result of crosslinking by DMS. By DTBP, which maximum distance is nearly equal with that of DMS (11.0 Å), only dimers were formed. But our results with crosslinkers longer than 11 Å indicate that his conclusion is not the case. And our results well coincide with those of gel filtration, sedimentation equilibrium and sedimentation velocity measurements by Schechter et al (17). Although from failures of crosslinking of any molecules we can not conclude that they are not associated in situ, the results that bands 1 and 2 polypeptides can be crosslinked only into 1-2, but not to 1-1 and 2-2 by both DTBP and other types of reagents, favor the interpretation that they are present in a pairwise manner. And the results can be interpreted as follows: higher temperatures and divalent cations promote the aggregation of these pairs into multimers. Even at 25 °C, at least some part of spectrin is aggregated into higher forms such as tetramer or more. Deoxycholate and Triton X-100 dissociate multimers into dimers, but do not dimers into monomers. The distance between adjacent, paired bands 1 and 2 polypeptides is considered to be close enough that longer crosslinkers may be unsuitable for their coupling into dimer. On the other hand, in multimers these pairs may be separated in some distance each other. And crosslinking between unpaired polypeptides may favor to longer crosslinkers and not to shorter ones. The length of DTBP and DTBE may just correspond to these requirements.

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