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The Use of Bifunctional Biotinyl Compounds to Determine the Arrangement of Subunits in Avidin

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A series of bisbiotinyl diamines was synthesized with between 9 and 25 bonds between the carboxyl groups of the two biotin residues. It was found that only one of the two biotin residues could combine with avidin when there were fewer than 12 bonds between the biotin residues. Compounds with longer chains behaved in a bifunctional manner and gave rise to linear polymers of avidin, which were characterized by electron microscopy and by gel filtration. The polymers formed with the shorter-chain reagents (12, 13 or 14 bonds) were relatively unstable and could be depolymerized by weakly bound analogues of biotin. The polymers of longer-chain reagents were not depolymerized under these conditions and were only slowly affected by added biotin. When the chain length of the reagent reached 23 bonds the polymers became much shorter, suggesting that the reagent was now able to link two subunits within the same avidin molecule. From the morphology of the polymers it could be concluded that the four subunits of the avidin molecules were arranged with 222 symmetry and that they were grouped in two pairs at opposite ends of the short axis of the molecule whose dimensions were 55 Å × 55 Å × 41 Å.

The experiments described in this paper arose from an interest in the possibility of determining the arrangement of the four subunits in the avidin molecule by electron microscopy. Although individual molecules of avidin (mol.wt. 63000; Green & Toms, 1970; de Lange, 1970) were clearly visible in negative stain, no significant subunit structure could be resolved. This is not surprising, since the smallest proteins in which subunits have been resolved by electron microscopy are aldolase (Penhoet, Kochman, Valentine & Rutter, 1967) and immunoglobulin G (Valentine & Green, 1967), in which both the molecule and its subunits are two or three times as large as those of avidin. It was argued that suitable bifunctional biotin compounds could join the tetramers by their binding sites and that a polymer would be produced whose morphology would give the desired information about the symmetry of the arrangement of subunits in the tetrameric molecule. There are only two basic types of symmetry possible for a molecule containing four identical asymmetric subunits in equivalent environments (Caspar, 1962), which may be called briefly twofold and fourfold symmetry. In the first, the tetramer can be generated from the monomer by the operation of three mutually perpendicular twofold rotations, whereas in the second it results from the action of a single fourfold rotation.

The choice of bifunctional reagent was not difficult since it was known that the carboxyl group of biotin could be linked to a variety of molecules, including native proteins, without impairing the binding by avidin (e.g. Green, 1963*b*). Any link of sufficient length between the carboxyl groups of two biotin molecules should provide a reagent of the desired type. Since polymethylenediamines were commercially available with any chain length between 2 and 12 methylene groups, their bis-*N*-biotinyl derivatives were synthesized for this work. Longer linking chains were synthesized by replacing the biotin by 4-*N*-biotinamidobutyric acid.

The three primary questions that we wished to answer with the aid of these reagents were as follows: (1) How long does the linking chain have to be before both biotins can be bound by avidin? (2) Are the products polymeric or monomeric and what is their morphology? (3) How does the stability of the products depend on the separation of the biotin residues? The stoicheiometry of the reaction with avidin was followed by spectro-

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photometric titration by using the dye 4-hydroxyazobenzene-2′-carboxylate as an indicator for vacant binding sites (Green, 1970). The amount of polymeric material formed was determined by gel filtration and the morphology of the polymers was studied by electron microscopy.

MATERIALS AND METHODS

Avidin was purified as described by Green & Toms (1970); the crystalline material used in these experiments bound 15μg of biotin/mg. D-Biotin was obtained from Roche Biochemicals, Welwyn Garden City, Herts., U.K. Polymethylenediamines were commercial products (Koch-Light Laboratories Ltd., Colnbrook, Bucks, U.K., for 7, 8, 10 and 12 methylene groups, and R. N. Emanuel, Wembley, Middx., U.K., for 9 and 11 methylene groups) and were used without further purification. Triethylamine was dried over NaOH and redistilled. Methyl chloroformate was redistilled. Tetrahydrofuran was stored over NaOH, dried with sodium hydride and distilled immediately before use. Dimethylformamide (fraction boiling at 150–151°C) was passed through a column of dry Zeo-Karb 225 (H^+ form; coarse mesh) to remove dimethylamine and dried overnight with calcium oxide. It was finally distilled *in vacuo* and stored over a molecular sieve (Union Carbide type 4A, 8–12 mesh). Bisbiotinyl compounds were synthesized by the mixed-anhydride method (Knappe, Brümmer & Biederbick, 1963).

4-N-*Biotinamidobutyric acid.* D-Biotin (250mg, 1mmol) was dissolved in dimethylformamide (9ml) in a three-necked flask fitted with stirrer, dropping funnel and drying tube. The solution was cooled to −15°C and triethylamine (0.152ml, 1.1mmol) was added with stirring, followed by slow addition of methyl chloroformate (0.106ml, 1.1mmol) in tetrahydrofuran (1.0ml). After 10min a suspension of 4-amino butyric acid (205mg, 2mmol) in a mixture of dimethylformamide (5ml) and triethylamine (0.42ml) was added. The temperature was allowed to rise and the mixture was stirred for 45min at room temperature. The solvents were removed *in vacuo* and the residue dissolved in warm aq. 20% (v/v) ethanol. The residue was filtered off and the filtrate acidified to pH2. The crystalline precipitate (264mg) was filtered off, dissolved in water with a minimum amount of 2M-Na_2CO_3 and reprecipitated with 2M-HCl. The product was twice recrystallized from hot water. After drying *in vacuo* the product was very hygroscopic and the analyses indicated the presence of small amounts of water (Calc. for $C_{14}H_{22}N_3O_4S_2$: C, 51.0; H, 7.0; N, 12.7. Found, C, 50.5; H, 7.1; N, 12.1%).

1,6-*Bisbiotinamidohexane and its homologues.* Biotin (200mg, 0.82mmol) was dissolved in dimethylformamide (7.5ml) and converted into the mixed anhydride with methyl chloroformate as described above. To the reaction mixture at −15°C was added triethylamine (0.114ml, 0.82mmol) followed by 1,6-diaminohexane (48mg, 0.41mmol) in dimethylformamide (1ml). A translucent solid separated during stirring for 45min at room temperature. The whole mixture was evaporated to dryness *in vacuo* and the residue dissolved in water at 70–80°C with the addition of a little ethanol. The solution was cooled slightly and the pH adjusted to 7–8. Soft crystals formed on further cooling (180mg). They were recrystallized from ethanol. The other bisbiotinyl compounds were synthesized in the same manner with minor modifications. With the shorter diamines (seven, eight or nine methylene groups) the reaction mixture was evaporated to dryness, the product was washed with 1M-HCl, 3% (w/v) $NaHCO_3$ and water and then crystallized from 95% (v/v) ethanol. The products from the longer diamines precipitated out from the reaction mixture and were filtered and washed as described above. All products were recrystallized several times from the solvents listed in Table 1. They did not crystallize well and usually formed spherulites, which were difficult to free from adhering solvent and which did not melt below their decomposition points. The analytical results are summarized in Table 1.

Bisbiotinamidobutyryl derivatives of diaminohexane, diaminodecane and diaminododecane. The coupling was carried out as described above, biotin being replaced by an equimolar amount of 4-biotinamidobutyric acid. The reaction mixture was evaporated *in vacuo* and the product was dissolved in hot dimethylformamide (4ml at 70°C). After addition of ethanol (4ml) and water (18ml) the pH was adjusted to neutrality and the product left to crystallize. The gelatinous spherulites were filtered and washed with 1M-HCl, 3% $NaHCO_3$ and water. The dried products were crystallized from the solvents shown in Table 1.

The bisbiotinyl compounds had very low solubilities in most solvents. Solutions in ethanol of sufficient concentration (1–2mM) for titrations could be prepared only with the lower homologues but solutions in dimethylformamide were more convenient and could be obtained with all the compounds.

Measurement of avidin concentration and biotin-binding activity. Avidin concentration was determined spectrophotometrically at 282nm by using $E^{1\%}_{1cm}$ 15.4 (Green & Toms, 1970), with a Unicam SP.700 recording spectrophotometer.

Biotin-binding activity was measured by spectrophotometric titration, by using the dye 4-hydroxyazobenzene-2′-carboxylate (Green, 1970) as an indicator for free binding sites. The combination of bisbiotinyl compounds with avidin was followed by the same method. A stock solution of avidin (0.2ml, 4mg/ml) was diluted in a 1cm cuvette with 0.1M-sodium phosphate buffer, pH7.0 (2.5ml). After determination of E_{282}, the dye solution (50μl, 0.01M) was added and E_{500} was measured. Portions (1–2μl) of the solution of bisbiotinyl reagent (1 or 2mM, in dimethylformamide) were added slowly from a Hamilton micro-syringe with continuous stirring. This was effected without removing the cuvette from the spectrophotometer, by employing a small battery-driven electric motor mounted on the thermostat-controlled cell housing. The E_{500} was recorded after each addition until a constant reading was obtained and titration curves were plotted to determine the end point.

Separation of polymers from monomer by gel filtration. This was carried out at room temperature on a column (1cm × 70cm) of Sephadex G-100, by using a peristaltic pump and a time-operated fraction collector. Polymer solutions for gel-filtration experiments were prepared by addition of a 2mM solution of the reagent in dimethylformamide to a well-stirred solution of avidin (5mg/ml in 0.2M-sodium phosphate buffer, pH8). When the maximal amount of polymer was desired 1 equivalent of the

Table 1. *Nomenclature and analytical results for bisbiotinyl diamines*

The bisbiotinyl compounds are characterized in the text as B*n* where *n* is the number of bonds separating the carboxyl carbonyl groups of the two biotinyl residues. For a bisbiotinamidoalkane *n* is equal to three more than the number of methylene groups in the diamine from which it was synthesized. This system has the advantage that the maximal separation of carboxyl groups of the biotins is close to 1.25 times this number, since most of the bonds are C–C single bonds, each of which contribute 1.25 Å to the length of a fully extended chain. The $-NH-CH_2-$ and $-\overset{O}{\overset{\|}{C}}-NH-$ bonds are only slightly shorter, contributing 1.16 Å and 1.18 Å respectively to the chain length. The length of the linking chain is given within 1% by the expression $(1.25n-0.3)$ Å.

Bisbiotinyl derivative of:	Abbreviation	Recrystallized from	Analytical results	%C	H	N
1,6-Diaminohexane	B9	95% Ethanol	Found	53.2	7.8	14.1
			Calc. for $C_{26}H_{44}N_6O_4S_2H_2O$	53.1	7.8	14.3
1,7-Diaminoheptane	B10	80% Ethanol	Found	54.3	8.3	13.8
			Calc. for $C_{27}H_{46}N_6O_4S_2H_2O$	54.0	8.0	14.0
1,8-Diamino-octane	B11	95% Ethanol	Found	54.4	8.2	13.4
			Calc. for $C_{28}N_{48}N_6O_4S_2H_2O$	54.6	8.1	13.7
1,9-Diaminononane	B12	Butan-1-ol	Found	55.3	7.9	13.0
			Calc. for $C_{29}H_{50}N_6O_4S_2H_2O$	55.4	8.3	13.4
1,10-Diaminodecane	B13	2-Methylpropan-1-ol	Found	56.8	8.8	12.8
			Calc. for $C_{30}H_{52}N_6O_4S_2H_2O$	56.1	8.4	13.1
1,11-Diaminoundecane	B14	Butan-1-ol–ethanol	Found	57.1	8.8	12.4
			Calc. for $C_{31}H_{54}N_6O_4S_2H_2O$	56.7	8.5	12.8
1,12-Diaminododecane	B15	Dimethylformamide–water	Found	57.6	9.1	12.2
			Calc. for $C_{32}H_{56}N_6O_4S_2H_2O$	57.4	8.7	12.5
1,6-Bis(4-aminobutyramido)hexane	B19	Butan-1-ol and ethanol–ether	Found	55.0	8.4	14.6
			Calc. for $C_{34}H_{58}N_8O_6S_2–C_2H_5OH$	55.1	8.2	14.3
1,10-Bis(4-aminobutyramido)decane	B23	Ethanol–ether	Found	57.5	8.9	13.1
			Calc. for $C_{38}H_{66}N_8O_6S_2–C_2H_5OH$	57.2	8.5	13.3
1,12-Bis(4-aminobutyramido)decane	B25	Ethanol–ether	Found	57.7	8.9	12.5
			Calc. for $C_{40}H_{70}N_8O_6S_2–C_2H_5OH$	58.1	8.8	12.9

reagent was added slowly from a Hamilton micro-syringe. For minimal polymerization 2 equivalents of the reagent (1 mol/binding site) were added rapidly. After being left for 30 min the samples were loaded on the column.

Electron microscopy. Negatively stained preparations of avidin and its polymers were made on freshly stripped carbon film (Valentine, Shapiro & Stadtman, 1968). The repeat distance of the polymers was measured on the original micrographs, by using glutaraldehyde-fixed catalase crystals (Wrigley, 1968) for calibration. Fields were selected containing both crystals and polymers. The lengths of straight sections of polymer were measured on a comparator and the mean of a total of 100 repeats was determined. Plates were taken at a magnification of ×55000 with a Phillips E.M. 200 microscope.

RESULTS

Synthesis and titration of bisbiotinyl compounds. The yields of the crude products were good, but considerable losses were experienced in the subsequent purification, because of their poor solubility in most solvents, from which they invariably precipitated as spherulites rather than true crystals. Most of the products retained tenaciously a molecule of water of crystallization, even when the proportion of water in solvent was low, and this was not removed by prolonged drying at 100°C *in vacuo*. The analytical values showed only moderate agreement with theory. With compounds B12, B14 and B15 some of the problems encountered may have been caused by impurities in the diamines used to prepare them. After the work had been completed the purity of each diamine, from the heptamethylene to the dodecamethylene compound, was checked by g.l.c. of the trifluoroacetyl derivative. The results are shown in Table 2. Considerable amounts of impurity, which behaved as shorter-chain homologues of the main component, were present in the longer-chain diamines. These homologues were presumably present in the resulting bisbiotinyl derivatives and could account for some minor anomalies in the succeeding results.

The equivalent weights of the compounds titrated against avidin were, with one exception (compound B13), in good agreement with either 1.0 or 2.0 available biotin residues per molecule (Table 3). The intermediate behaviour of compound B13, also illustrated in Fig. 1, is discussed below. Titration curves of representative compounds are shown in Fig. 1, where they are compared with that given by biotin and with a theoretical curve for a bifunctional compound. The short-chain compounds B11 and B12 showed almost identical behaviour, similar to that of biotin except for the initial part of the curve, where some bifunctional binding was indicated. Over most of the curve each molecule of reagent displaced only one molecule of dye. An increase of only two methylene groups (2.5 Å) in chain length permitted each molecule of compound B14 to displace 1.9 molecules of dye over the first three-quarters of the curve. Compound B15 was similar and the longer-chain reagents were bifunctional over the whole curve.

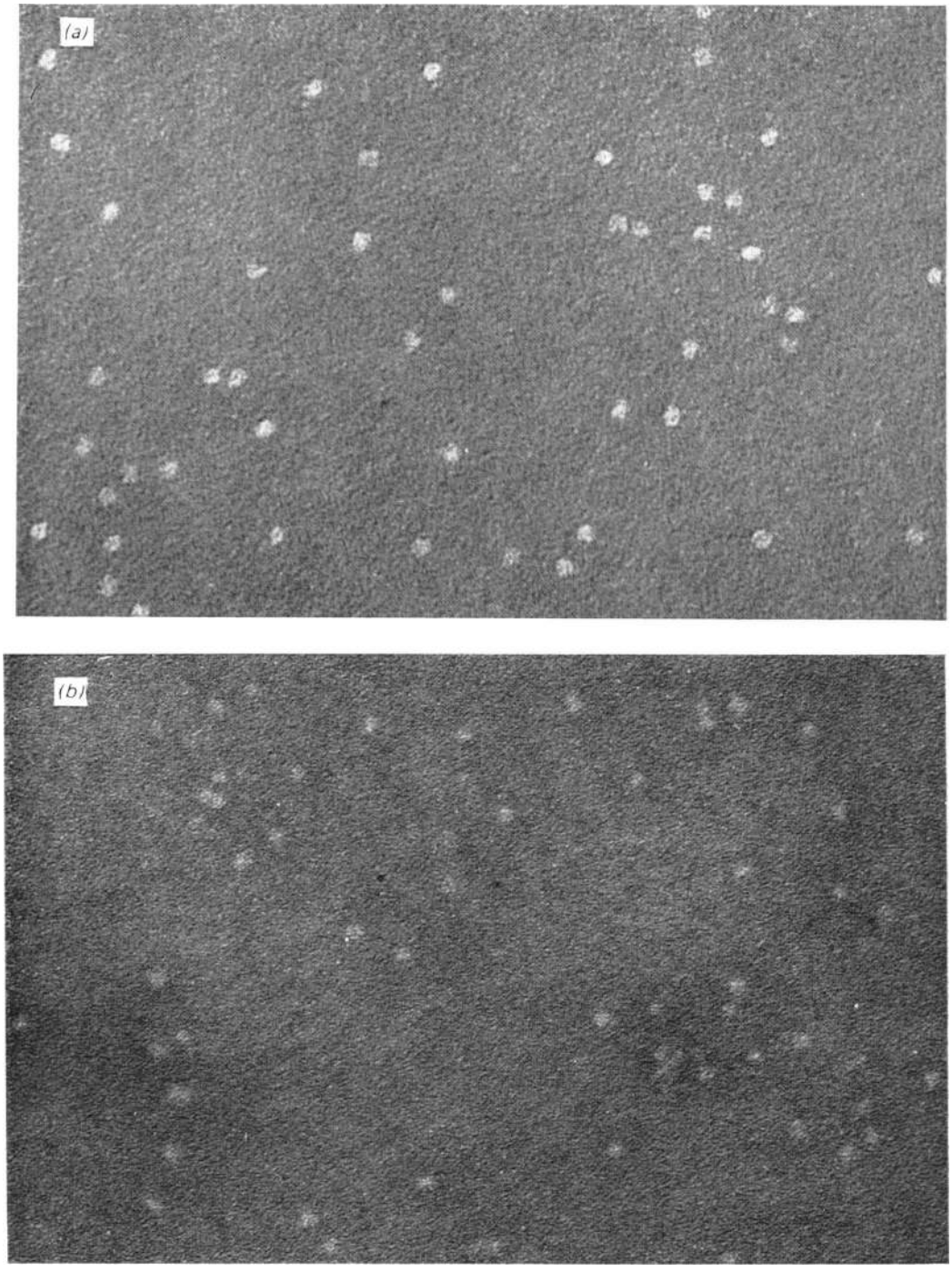
Electron microscopy of avidin and its polymers. Avidin by itself (Plate 1*a*) appeared as a uniform population of polygonal, sometimes almost rectangular profiles, with one dimension usually about 10% shorter than the other. The slight difference in the two dimensions is of doubtful significance and could be due to particular orientations of equidimensional molecules on the film. We have therefore assumed this profile to be equidimensional (55 Å × 55 Å). Occasional molecules with one much shorter dimension (about 40 Å) were observed, which in the light of later experiments appears to represent a second profile of the molecule (40 Å × 55 Å). The occasional suggestion of subunit structure was not consistently observed and was probably due to phase artifacts (Haydon, 1968).

There was no detectable difference between avidin and the avidin–biotin complex, which was not surprising in view of the lack of effect of biotin on other physical parameters of the molecule (Green, 1963*c*). The products of reaction with the short-chain bisbiotinyl compounds (see e.g. Plate 1*b*) did not differ in appearance from avidin apart from the presence of a very few dimers and trimers. It was noteworthy that whereas both avidin and the avidin–biotin complex gave a good coating of molecules on the carbon film at very low protein concentrations (4 μg/ml), the complexes of avidin with the short-chain reagents (compounds B9,

Table 2. *Characterization of polymethylene diamines*

These analyses were provided by Dr D. H. Calam. Trifluoroacetyl derivatives were prepared with 50% (w/v) trifluoroacetic anhydride in ether at room temperature. Excess of reagents were removed *in vacuo* and the derivatives, dissolved in ethyl acetate, were analysed on an OV-17 silicone support at 190°C in a Perkin–Elmer F.11 gas–liquid chromatograph.

Nominal chain length of diamine	7	8	9	10	11	12
Content of nominal diamine (%)	99.5	99.5	95	98	85	90
Chain lengths of contaminants			8, 10, 11	7, 9	5 or 6, 7, 8, 10	7, 8, 9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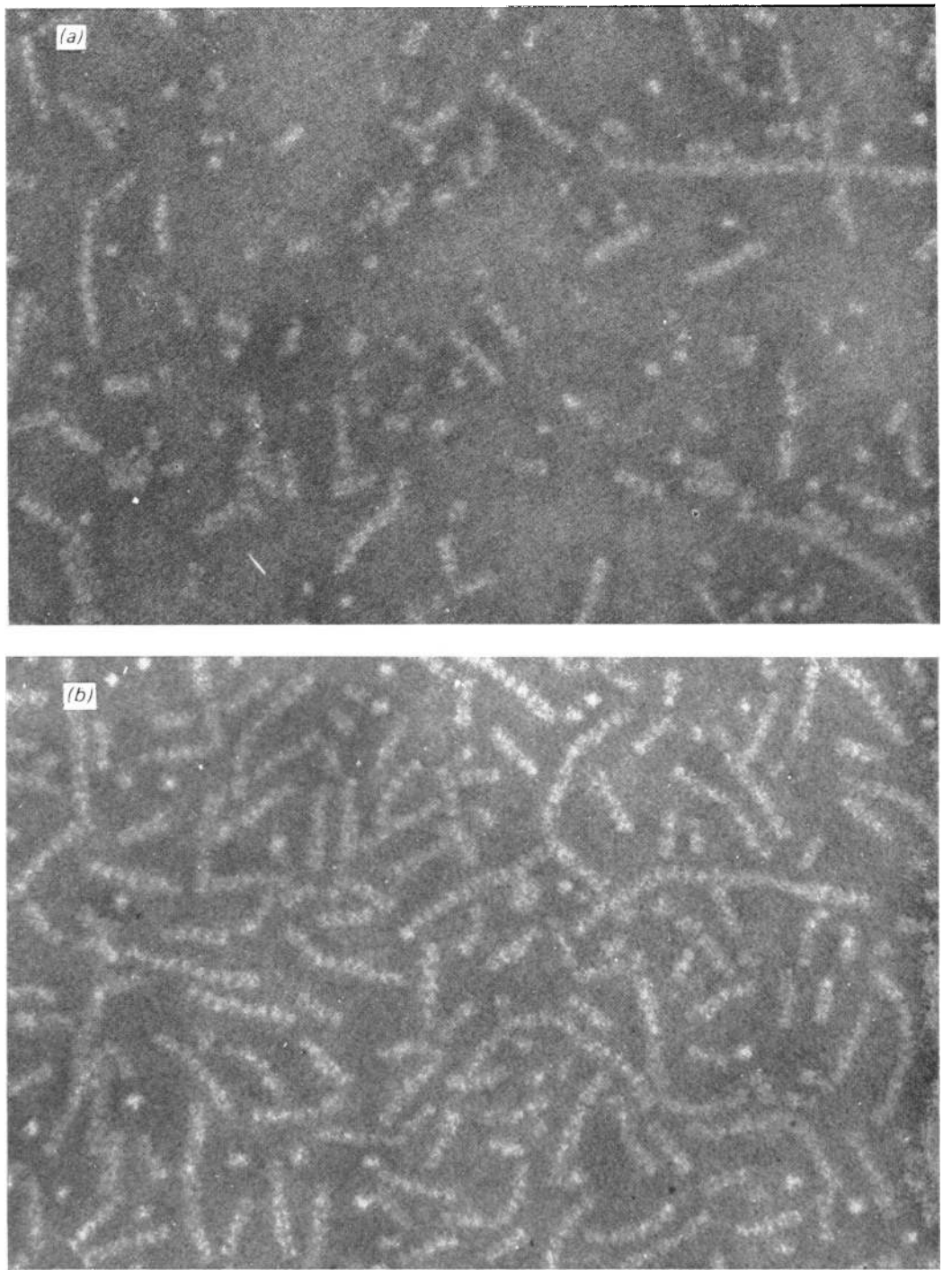
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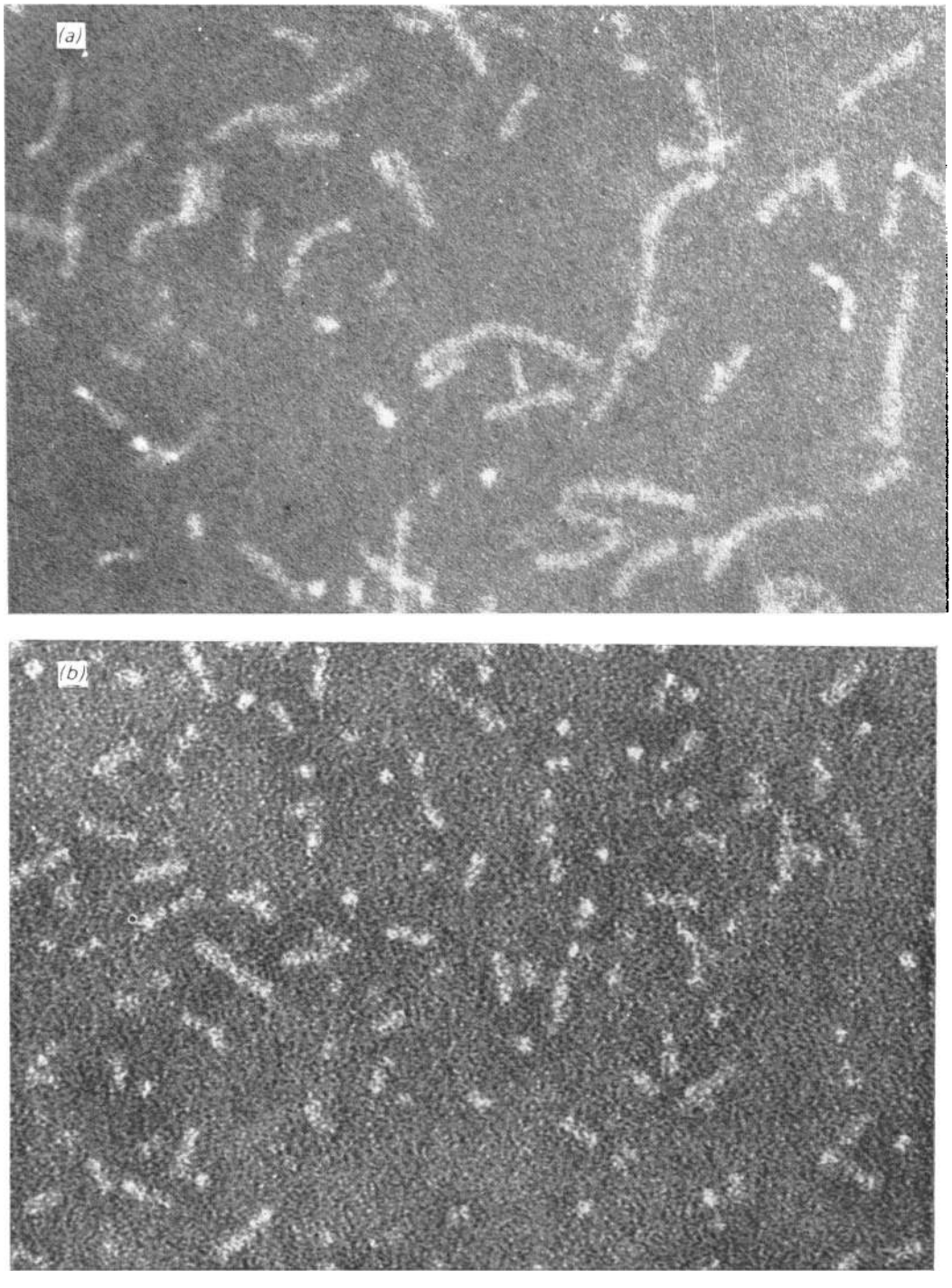
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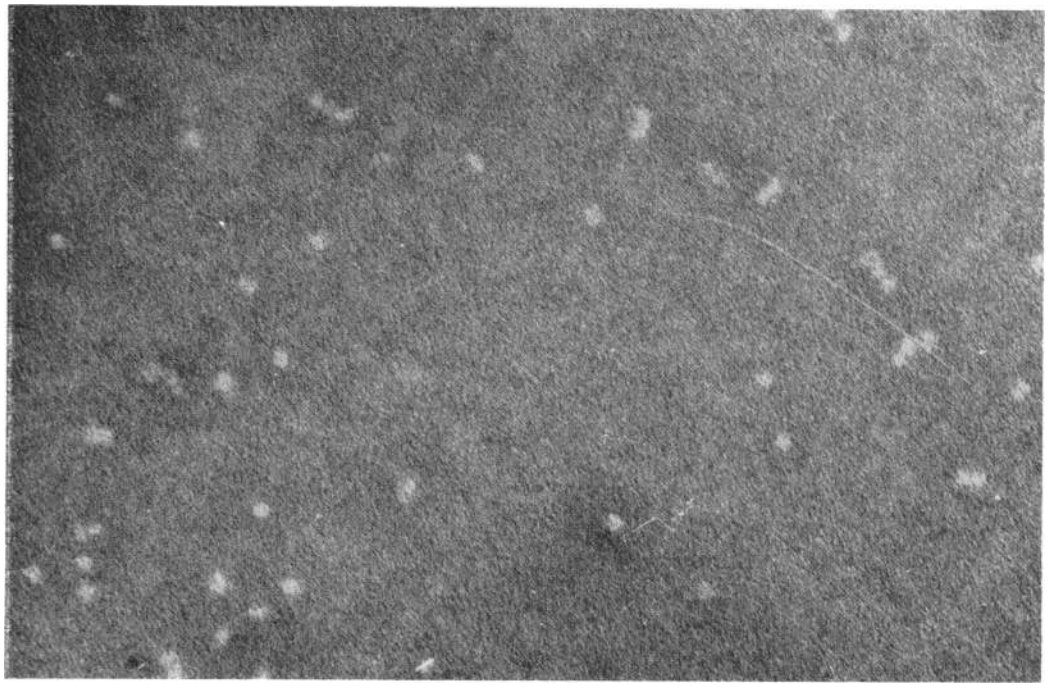

EXPLANATION OF PLATES 1, 2, 3, AND 4

Electron micrographs of avidin and its polymers. The molecules were adsorbed on freshly stripped carbon film (Valentine *et al.*1968), from a solution of the concentration indicated. The preparations were negatively stained in 2% (w/v) sodium silicotungstate and examined in a Philips E.M. 200 electron microscope at a magnification of × 55000. The final magnification of all the plates was × 500000. 1(*a*), Avidin (10 μg/ml); 1(*b*), avidin (150 μg/ml), +compound B11; 2(*a*), avidin (30 μg/ml)+compound B12; 2(*b*), avidin (30 μg/ml)+compound B15; (note the occasional branch points); 3(*a*), avidin (30 μg/ml)+compound B19; 3(*b*), avidin (30 μg/ml)+compound B23; 4, avidin (100 μg/ml)+compound B25.

Table 3. *Chain length of bisbiotinyl diamines and stoicheiometry of their reaction with avidin*

Full names of bisbiotinyl derivatives are given in Table 1. The number of moles of reagent was determined by extrapolation of the linear portion of the titration curves (Fig. 1).

Bisbiotinyl derivative	B9	B10	B11	B12	B13	B14	B15	B19	B23	B25
Number of bonds (n) between biotin carboxyl groups	9	10	11	12	13	14	15	19	23	25
Distance in Å between carboxyl groups ($1.25n-0.3$)	10.9	12.2	13.4	14.7	15.9	17.2	18.4	23.4	38.4	30.9
Mol of reagent required to displace 4 mol of dye from avidin	4.32	4.00	3.72	3.72	2.40	2.16	2.16	2.04	2.08	1.88

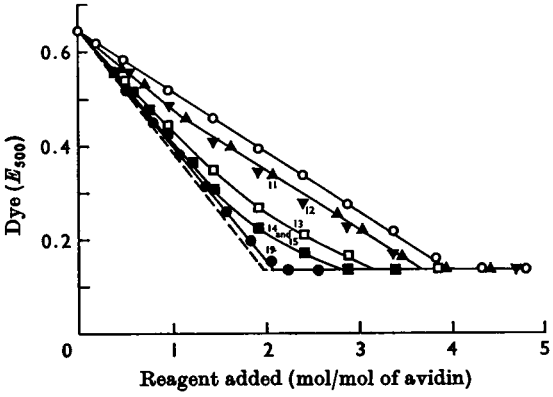

Fig. 1. Displacement of dye from avidin by bisbiotinyl reagents. The numbers give the number of single bonds between biotinyl residues. ○, Biotin; ▲, compound B11; ▼, compound B12; □, compound B13; ■, compounds B14 and B15; ●, compound B19. The points for compounds B14 and B15 lay so close together that only one set has been included. Complete displacement of the dye by 2 mol of reagent/mol of avidin indicates fully bifunctional behaviour (----).

B10 and B11) required the use of 30–50-fold higher concentrations to obtain adequate coating of molecules (Plate 1*b*). No such problem was encountered when polymeric species were present, but depolymerization (e.g. of the B14 polymer) by an excess of reagent gave monomeric species which again adsorbed only with difficulty.

When the number of methylene groups was increased from eight to nine (compound B11 to compound B12) most of the avidin molecules aggregated to give linear polymers (Plate 2*a*). It appeared likely that the discrepancy between this result and that of the dye titration, which suggested that compound B12 was monofunctional, was caused by the presence of the dye in the titration experiment. This was confirmed by addition of 0.5 mM dye to the polymers given by compound B12. This led to rapid depolymerization, showing that the second biotin residue of the reagent was not sufficiently exposed to bind firmly to another avidin molecule and displace the dye, the dissociation constant of which is 5.8 μM (Green, 1970). Partial depolymerization by the dye could also explain the intermediate titration behaviour of compound B13 (Fig. 1). All the longer-chain reagents gave similar polymers (Plates 2, 3 and 4), though both the amount and the degree of polymerization was decreased with the longest linking chains (Plates 3*b* and 4).

The width of the polymers (55 Å) was the same as that of single avidin molecules. The subdivisions between neighbouring molecules were usually resolved so that the repeat distance along the chain (40–45 Å) could be accurately measured. Since the four binding sites of avidin were occupied by the bifunctional reagent and since branch points were rare, each molecule was doubly linked to its neighbours. Terminal molecules may have had vacant sites or sites blocked either by small amounts of biotin (present in the avidin) or by the bisbiotinyl compound bound intramolecularly. It seems that this last effect became important only with the two longest-chain reagents B23 and B25, which gave predominantly short polymers (Plates 3*b* and 4). Once intramolecular binding became possible it should have been favoured over polymerization because of the increased translational entropy of the monomeric product. These results would therefore indicate that little or no intramolecular binding was possible unless the linking chain between the biotin residues was longer than 20 Å (compound B19). The observation that the repeat distance was significantly less than the width of the polymers confirms the suggestion made above that avidin has at least two distinct profiles: an approximately equidimensional 55 Å × 55 Å one, seen predominantly when single molecules are examined, and the 55 Å × 40 Å profile exhibited in the polymers.

The relation between repeat distance and the

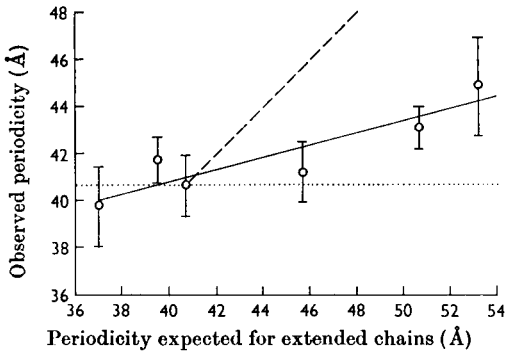

Fig. 2. Periodicity of avidin polymers as a function of the chain length of the bisbiotinyl reagent. Measurements were made on a total of 100 monomers linked in long or short chains. The limits of error indicated are the standard deviations of the periodicities of single molecules of polymer. The individual polymers measured were, except for some trimers of compound B25, at least tetrameric and usually longer. The periodicity expected for extended chains of compound B15 (abscissa) has been set equal to the observed value (ordinate) on the assumption that the avidin molecules are just held in contact by the fully extended chain of compound B15, since this is the shortest chain to give polymers that are not rapidly depolymerized by biotin. The dotted line (·····) shows the relation expected for rigid avidin molecules when maintained in contact by weak interactions. The dashed line (-----) shows the result that would be given by fully extended chains in the absence of such interactions.

length of the linking chain was approximately linear within the limits of error of the measurements (Fig. 2). The increase in periodicity was only one-quarter of that which would have been expected if the chains linking the avidin molecules together had been fully extended (-----). It would be surprising if the relationship were truly a linear one, since there are several independent factors that could influence the periodicity, some of which will be considered below. Here, it may be pointed out that the results could also be taken to show little or no significant influence of chain length on repeat distance with the shorter-chain reagents (B12-B19), followed by considerable increases with the two longest-chain reagents (B23 and B25). This could be caused by attractive forces between neighbouring avidin molecules sufficient to maintain them in contact, until prevented by the bulky linking chains of compounds B23 and B25.

Gel filtration of avidin polymers. Electron microscopy by the methods used here can give only a qualitative picture of the molecular species present in solution, since it cannot be assumed that all molecules adsorb on the carbon film at the same rate. The considerable difference in behaviour of avidin and avidin monomers saturated with bifunctional reagent emphasizes the pertinence of this consideration. Gel filtration was therefore used to obtain a quantitative measure of the amount of polymer formed and to measure the depolymerization produced by an excess of the reagent and hence to provide some indication of the relative stability of the different polymers. Since it was not possible to resolve members of the series of polymers from each other it was decided to use a relatively coarse fractionation on Sephadex G-100 to separate monomer from total polymer. An approximate estimate of the amount of dimer in the system could also be made. The fraction of monomer was determined both in the presence of 2 mol of reagent/mol of avidin and on a parallel sample to which 4 mol of reagent/mol of avidin had been added. The presence of 1 mol of reagent/binding site in the second type of experiment would be expected to lead to extensive depolymerization of the polymers only when the binding of the second biotin group of each molecule of reagent was considerably weaker than that of the first, since, in the absence of steric interference, biotin derivatives are bound so firmly that their replacement by another biotin compound requires several weeks (Green, 1963*a*), rather than the minutes or hours involved in these experiments.

The elution profiles of the products formed with compound B14 are shown in Fig. 3(*a*). About 90% of the avidin was polymerized in the presence of 2 mol of the reagent, but only 19% when 4 mol was added/mol of avidin. Thus although these B14 polymers were stable in the presence of the dye used for titrations they were largely broken down by excess of bifunctional reagent. The results of a similar pair of columns with the longest-chain compound, B25 (Fig. 3*b*), showed that even under optimum conditions for polymerization, with all the binding sites occupied by the reagent, a large proportion of the avidin was monomeric. Further, the polymer peak differed from that given by compound B14 in that it was not at the exclusion limit of the column but near the position expected for dimers of avidin. The proportions of monomer to polymer were not significantly altered by a twofold excess of the reagent, which was not surprising since the long-chain compounds are likely to be bound almost as firmly as biotin itself.

The results of all the gel-filtration experiments are summarized in Fig. 4, where the percentage of avidin as polymer is given as a function of the chain length of the reagent. The results of the columns run in the presence of 2 mol of reagent/mol of avidin confirm that conclusions drawn from the electron micrographs. No significant proportion of polymer was observed with reagents of chain lengths shorter than that of compound B12 and once polymerization was possible it went almost to completion. The

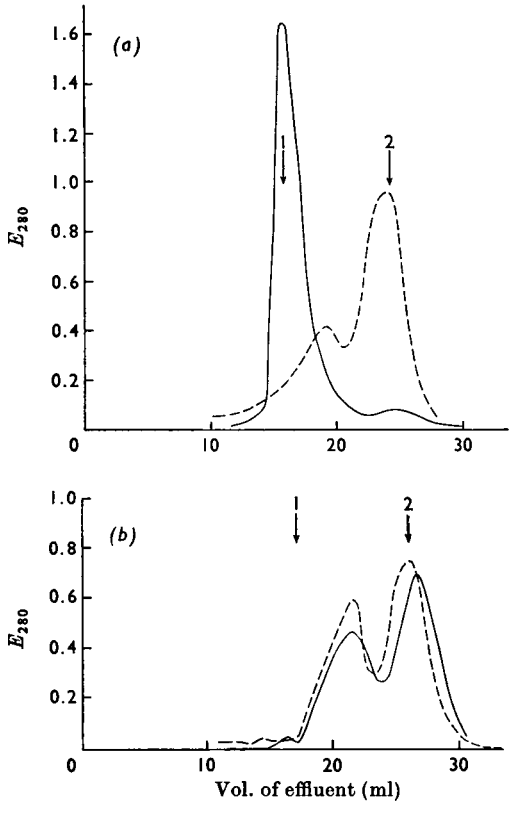

Fig. 3. Gel filtration of polymers of avidin, formed with compounds B14 (*a*) and B25 (*b*), on Sephadex G-100. The polymers were prepared from 2mg of avidin (0.12μmol) and either 0.06μmol (——) or 0.12μmol (----) of the bisbiotinyl reagent. The arrows indicate the void volume of the column (1) and the elution volumes characteristic of monomeric avidin (2).

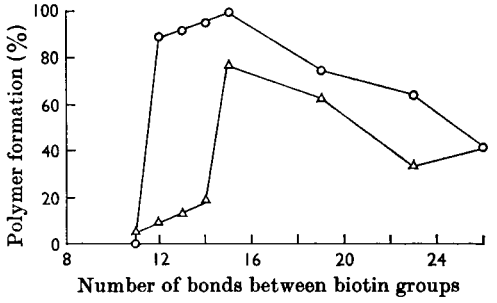

Fig. 4. Formation of polymers of avidin and its reversal, as a function of the chain length of the bisbiotinyl reagent. The percentage of polymer (including dimer) was determined from the areas under the curves obtained in experiments such as those shown in Fig. 3. ○, Avidin+2mol of reagent/mol; △, avidin+4mol of reagent/mol.

decrease in both amount and size of polymer as the chain length increased is clear from Figs. 3 and 4 and suggest that appreciable intramolecular binding was present from compound B19 onwards. All three of these long-chain compounds showed a marked shoulder on the polymer peak in a position corresponding to avidin dimers, which was not seen with the shorter-chain-length compounds. The amount of dimer formed with each reagent could only be estimated approximately, since the peaks were poorly resolved. The dimer accounted for about 75% of the polymer from compound B25, 50% of that from compound B23, and 40% of that from compound B19, but less than 15% of that from compounds B14 or B15. The polymers formed with the three short-chain reagents (B12, B13 and B14) were relatively labile and underwent extensive dissociation in the presence of excess of reagent. Further experiments with an excess of biotin, rather than of the very insoluble reagent, showed almost complete dissociation of these polymers. For example, only 5% of polymer remained from the 95% originally produced with compound B14. The B15 polymers were much more stable, the amount of polymer being decreased only to 77% with 4mol of reagent (or to 40% when incubated for 2h with a fivefold excess of biotin). A similar degree of reversal was seen with polymers from compounds B19 and B23, whereas those from compound B25 were not measurably depolymerized.

DISCUSSION

The results obtained by the three different techniques of dye titration, electron microscopy and gel filtration lead to essentially similar conclusions about the combination of avidin with the series of bisbiotinyl compounds. The only major discrepancy was that almost no polymer fraction could be detected with the compound B12 by dye titration, whereas extensive polymerization was indicated by both the other techniques. This was shown to be caused by competition between the dye used as an indicator and the second biotin group of each reagent molecule that was bound. This emphasizes that the bifunctional behaviour of a particular compound is not entirely intrinsic but is dependent on the method used to observe complex-formation. In principle one might also expect to find differences between electron microscopy and gel filtration since the protein concentrations used in the two methods may differ by one or two orders of magnitude. Electron microscopy as usually performed is not suitable for detecting weak complexes since it is essential to work at low protein concentrations

(10–40 μg/ml) to avoid difficulties of interpreting overcrowded fields. Complexes with dissociation constants greater than 0.1–1 μM are therefore unlikely to be detectable unless they can be stabilized by secondary covalent bonds before dilution for microscopy. The complexes formed here were of considerably greater stability than this and only one minor discrepancy between the methods, discussed below, was noted.

The conclusions from these experiments can be summarized as follows. When there are fewer than twelve bonds (15 Å) between the biotin carboxyl groups the second biotin moiety does not project far enough from the surface of the avidin to reach a binding site on another avidin molecule. When there are 12, 13 or 14 bonds (15 Å–17.5 Å) between the biotin moieties, weak binding of the second biotin moiety becomes possible and reversible polymerization is observed. Addition of one more methylene group (compound B15), allows the second biotin moiety to be bound almost as strongly as the first and the polymerization becomes only slowly reversible. The polymers found with the longer-chain reagents retained high stability but the amount decreased and the length was considerably less. Since the binding remained bifunctional this indicated intramolecular binding of the reagent.

The transitions between the different types of behaviour were fairly abrupt, as would be expected for a rigid system, but there were some gradual effects, which deserve comment. The relatively weak reversible binding observed with compound B12, B13 and B14 could have been caused either by the inability of the second biotin moiety to reach its normal binding site or by the requirement for compression of the protein surface in order that it should reach the normal site. In other words, either the second biotin moiety cannot form all the weak bonds necessary for firm binding or it can only form them at the expense of a considerable free energy of distortion. The following argument suggests that a combination of both effects may be involved. It is likely that compound B15, which has the shortest chain giving stable polymers, just holds neighbouring avidin molecules in contact with each other. If the surface of the avidin were incompressible then the repeat distance could not decrease much below 41 Å as the linking chain was shortened, so that the second biotinyl residue in the polymers linked by compound B12, for example, would be 3.7 Å removed from its normal binding site. The results in Fig. 2 show a shortening of the repeat distance by about 1 Å, which, if significant, would indicate a slight compression of the surface and a displacement of the second biotin moiety of compound B12 by 2–3 Å from its normal location. In view of the scatter of the results in Fig. 2, this argument is only tentative, but it is useful in that it provides some indication of the factors that affect the repeat distance. Such a displacement of the biotin residue from its normal site would lead to the breakage of a number of weak bonds and to a considerable loss of stability. The free energies of binding of the second biotinyl residues of compounds B12, B13 and B14 (4.6, 5,9 and 7.1 kcal/mol) were calculated from the results of competitive binding experiments with the indicator dye (N. M. Green, unpublished work).

There is some doubt about the chain length at which intramolecular bonding became possible. The gel-filtration results showed a significant increase in the amounts of monomer and dimer with compound B19, although the length of the polymers seen in the electron microscope (Plate 3*a*) was not much decreased. The gel-filtration result is probably the more reliable since the technique looks impartially at all species, whereas electron microscopy examines only those molecules adsorbed on the carbon film. Since the results with compound B11 and shorter chains suggested that avidin monomers with projecting bifunctional reagent were rather poorly adsorbed this could at least partially account for the discrepancy between the two techniques.

Another anomaly to be explained is the partial depolymerization of the more stable polymers by excess of bifunctional reagent. In an ideal system one would have expected that if the polymerization were reversible then excess of reagent should have taken the system back to monomers. Three possible explanations for partial depolymerization are: heterogeneity of the avidin, heterogeneity of the bifunctional reagent and slow equilibration in systems where the second biotin moiety is bound firmly. There have been indications of some polymorphism of avidin (Melamed & Green, 1963; Green & Toms, 1970) but the published sequence (de Lange & Huang, 1971) suggests the presence of only two species with a difference of a single amino acid residue. The presence of lower homologues in the bisbiotinyl reagents (Table 2) could account for part of the effect observed with compound B15, since the polymer from the B12 contaminant would have been depolymerized by the excess of reagent. Slow equilibration is a more likely general explanation, in view of the very slow exchange of biotin bound to avidin. For example, if, during the mixing of the reagents, polymers were to form during the addition of the first portion of the reagent to avidin, they might be only partially depolymerized by the excess of reagent during the 30 min preceding the loading of the column. It was not possible to eliminate this effect by adding the avidin to an excess of the reagent, because the solubility of the latter was too low. The best that

could be done was to minimize it by performing the addition of the reagent as rapidly as possible with a syringe and a magnetic stirrer. The results obtained in this way were reproducible, but it is possible that different mixing procedures would have produced different results. The amount of polymer obtained in these experiments therefore has only qualitative significance.

We can draw a number of secondary conclusions from these answers to the primary questions posed in the introduction and derive a model for the arrangement of subunits and the location of the binding sites in the avidin molecule. The observation that the polymers were linear and almost unbranched showed that the four subunits of the molecule were arranged with twofold symmetry. A molecule with fourfold symmetry could give the polymers illustrated in Fig. 5. Although linear polymers could be formed they would not be of the type shown in Plates 1–4. The binding sites are therefore on opposite sides of a molecule with 222 symmetry, whose overall dimensions are 55 Å × 55 Å × 41 Å. Since the minimum repeat distance of the polymers is 41 Å the binding sites must be located on the 55 Å × 55 Å surface. The symmetry and dimensions of the molecule agree well with those determined by X-ray crystallography (Green & Joynson, 1970). The molecular weight calculated from these dimensions was 100000 for a molecule with a square 55 Å × 55 Å profile or 78000 for one with a 55 Å-diameter circular profile. This shows reasonable agreement with the molecular weight of 63000 estimated from the chemical composition, taking into consideration the errors involved in estimating such small dimensions by electron microscopy.

It is also possible to draw tentative conclusions about the depth of the binding sites and their separation from each other, which are illustrated in Fig. 6. Before discussing this interpretation in detail it is worth considering the most appropriate definition of the surface of a protein, since one of the parameters we wish to consider is the depth of the binding sites relative to some reference surface. Since the bifunctional capability of the reagents is determined by the closest distance of approach of two suitably oriented avidin molecules, the most appropriate surface is a van der Waals surface defined by the contours of an elastic envelope stretched over the protein. This will be the same as the conventional van der Waals surface of the protein, defined with respect to collisions with small molecules, only if the surface is everywhere convex. Any large concavities would lead to the inclusion of significant amounts of solvent within the van der Waals envelope defined here.

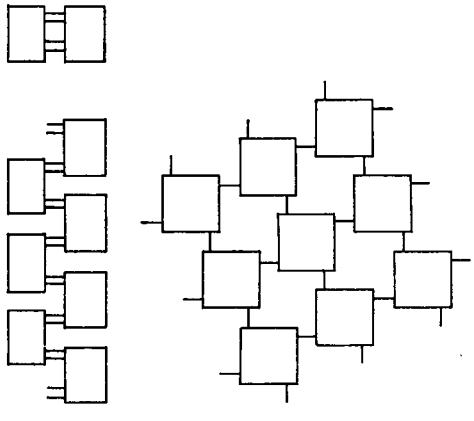

Fig. 5. Types of polymer that can arise from a molecule with fourfold symmetry.

In its normal state the carboxyl group of biotin is buried about 9 Å below the surface of the protein, since the shortest chain that permitted stable binding of both biotin residues was that of compound B15 in which the maximum separation of the two biotin carboxyl groups was 18 Å. In the strained state represented by the B12, 13 and 14 polymers this distance may be decreased by 2–3 Å.

When we come to consider the separation of the members of a pair of neighbouring sites it is not possible to draw such definite conclusions. It appears that intramolecular bridging is appreciable with compound B19, since the amount of polymer formed with this reagent was significantly less than was formed with compound B15, though the electron micrographs suggest that it did not become extensive before compound B23. If the polymethylene chain has to extend to the surface of the protein before it can bridge across to the other site, this result would imply that the entrances to the two sites must be within 6–10 Å of each other, a surprisingly small separation. If, however, there are clefts or depressions in the protein surface in appropriate regions, then the polymethylene chain could pass within the van der Waals surface and link together biotin carboxyl groups as much as 25 Å apart. Two different models are shown in Fig. 6, but a variety of possibilities exist with the sites separated by distances between 5 Å and 25 Å.

The localization of the biotin ring system with respect to the carboxyl group cannot be determined from these results. It is unlikely to be buried to the maximum depth of 10 Å, since the rapid reaction of avidin with biotin (Green, 1963*a*) indicates that the binding site must be readily accessible to molecules diffusing from the solvent. On the other hand the firm binding of biotin and of a wide variety of structural analogues (Green, 1963*b*) suggests that there is extensive interaction of the protein with all parts of the surface of the biotin, so that the whole

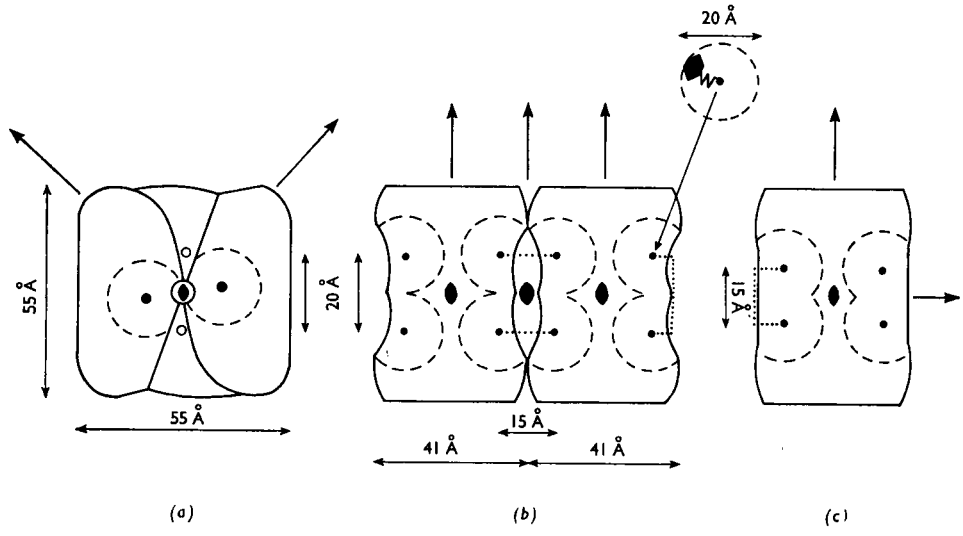

Fig. 6. Structure of avidin polymers. The carboxyl groups of the biotinyl residues are represented by either filled or open circles. The location of the remainder of the biotinyl residue with respect to the carboxyl group cannot be accurately defined by the present results and could lie anywhere within the 20 Å diameter spheres indicated by dashed lines (----). The chains linking the biotinyl residues are represented by ·····. (*a*) End view of a polymer chain; (*b*) and (*c*) side view of molecules in a polymer chain. (*a*) shows one 55 Å × 55 Å face of a terminal avidin molecule with its binding sites represented by filled circles. The pair of sites on the opposite 55 Å × 55 Å face on the underside of the terminal molecule are shown as open circles. Conventional symbols (→) and (◆) show the twofold symmetry axes which relate the sites to each other. The symmetry of the whole polymer is s22 (Klug, Crick & Wyckoff, 1958) indicating a twofold axis down the polymer axis, two mutually perpendicular twofold axes at right angles to this and a screw axis relating one molecule to the next. The amount of twist between one molecule and the next is determined by the angle between the lines joining pairs of sites on the upper and lower surfaces of the molecule. This angle is shown arbitrarily as about 70° in (*a*) and equally arbitrarily as 0° in (*b*) and (*c*). The angle of 0° was chosen to simplify the diagram and to bring all the twofold axes either into the plane of the paper or at right angles to it. As a result the side views (*b*) and (*c*) are not consistent with the end view (*a*), although either would be consistent with the experimental results. Two different side views are shown to illustrate two different separations of the sites. The 20 Å separation, (*b*), requires a deeper depression or cleft between the sites than does the 15 Å separation, (*c*).

of the ring system is likely to be buried in a cleft about 6–7 Å in depth. The maximal depth of the site relative to the surface is therefore about 15 Å.

This description of the gross morphology of the binding sites of avidin may be compared both with results obtained by the same method for the sites of anti-DNP antibodies (Valentine & Green, 1967) and with the more precise information available about the binding sites of proteins that have been studied by X-ray crystallography. Extension of the results of Valentine & Green (1967) (N. M. Green, unpublished work) has shown that a chain of five to six methylene groups between two dinitrophenylamino groups is the shortest that will allow both ligands to bind. The earlier estimate of eight methylene groups was too high. The amino groups of the ligand are thus only 3.5 Å below the surface of the protein and the maximum depth of the cleft would be about 10 Å.

It is often difficult to extract the corresponding information from published results of X-ray crystallographic investigations and it is usually necessary to make measurements on a model to obtain exact information. However, even without such measurements it is still possible to conclude that few of the ligands are located as far below the protein surface as is biotin. In general the most exposed part of the ligand is within 1–2 Å of the surface and the maximum depth of the site can be roughly estimated from the dimensions and orientation of the ligand. In myoglobin the distance from the haem carboxyl groups to the surface of the methyl and ethyl groups on the opposite side of the ring can be calculated from the published co-ordinates (Watson, 1969) to be about 15 Å. Similar estimates from the dimensions and orientation of bound tri-*N*-acetylglucosamine (Blake *et al.* 1967) give a depth of 7–8 Å for the cleft in lysozyme in the

neighbourhood of the catalytic centre. In lactate dehydrogenase, where the symmetry of the molecule is the same as that of avidin, the four coenzyme molecules are inserted into slots which, measured on a 5 Å resolution model (provided by Dr A. J. Wonacott, Molecular Biology Laboratories, Hills Road, Cambridge, U.K.), were no more than about 8 Å in depth in the most deeply buried region of the nicotinamide ring (Adams, McPherson, Rossmann, Schevitz & Wonacott, 1970).

Examination of the space-filling model of α-chymotrypsin suggested a parallel with the binding site illustrated in Fig. 6. This model shows a well defined substrate slot, 5.5–6 Å deep, into which the non-polar side chain of substrate fits (Steitz, Henderson & Blow, 1969). However, the slot is not on the outermost surface of the molecule but is at the bottom of a shallow depression about 7 Å deep and 20–30 Å across, so that the total distance from the van der Waals surface, as defined here, to the bottom of the slot is about 12 Å. If this provides any sort of analogy for the situation in avidin then the carboxyl group of biotin may be freely accessible to solvent in spite of its distance from the surface.

These conclusions are relevant to the interpretation of the inhibition of biotin-containing enzymes by avidin. Most biotinyl enzymes are irreversibly inhibited by avidin, suggesting that the biotin is in an exposed position, with the carboxyl group at least 9 Å from the surface of the protein. This is about 1.5 Å longer than the link provided by the side chain of lysine, to which the biotin is linked covalently and suggests that this lysine may itself be exposed, possibly in a flexible loop. This is consistent with the requirement, imposed by the carrier role of the biotin, for it to shuttle back and forth between catalytic sites on two different subunits of the enzyme (Moss & Lane, 1971). The only biotinyl enzyme known not to be inhibited by avidin is the polymeric active form of mammalian acetyl-CoA carboxylase (Ryder, Gregolin, Chang & Lane, 1967). Here the biotin must be in a somewhat more restricted situation, though it could still be quite exposed and yet be unavailable to avidin.

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