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Studies on the interaction of a surface-bound ligand with a multi-valent high-molecular-weight ligate

The biotinylcellulose–avidin system

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ABSTRACT

Two types of biotinylated cellulose disks were examined: filter-paper disks to which biotin had been covalently attached directly to the paper surface (biotinylcellulose) and disks on which biotin was attached to polyacrylamide side-chains grafted onto the filter-paper surface (biotinylpolyacrylamide–cellulose). The amount of avidin taken up from solution by these disks was linearly related to the avidin input concentration and could be estimated by exposure to [^{14}C]biotin. The avidin-binding capacity of the disks depended on the surface density of covalently attached ligand and exhibited hyperbolic, Langmuir-type behaviour for both types of disks. The [^{14}C]biotin binding capacity of avidinylated disks, on the other hand, showed anomalous, biphasic behaviour: at higher ligand densities, a decrease in [^{14}C]biotin binding was observed. The largest anomalies were obtained with biotinylpolyacrylamide–cellulose disks. Calculated ratios of bound [^{14}C]biotin *vs.* amount of avidin tetramer (B/A_4) showed a similar biphasic behaviour. A constant value of $B/A_4 = 3$ was obtained at low ligand densities, whereas B/A_4 decreased monotonously with increasing ligand density and asymptotically approached $B/A_4 = 1$. The data could be explained by assuming that at high ligand densities tetrameric avidin interacts with more than one surface-bound biotin residue.

INTRODUCTION

The specificity and ease of operation of solid-phase affinity systems has fostered their widespread use in the analysis and separation of both large and small biomolecules[1–8]. However, there are numerous instances where such systems are reported to exhibit severe functional anomalies. Mainly these entail failures in recognition, where

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the soluble ligand does not bind to a support to which it ought to bind. Alternatively, specifically bound ligands cannot be eluted under reasonably mild conditions[9-11]. This paper deals with mechanisms underlying phenomena of this nature.

In previous papers we described a simple, rapid assay for biotin based on filter-paper disks modified to contain covalently attached biotin[12,13]. The assay involves sequential exposure of these biotinylated disks to avidin and biotin as indicated in Fig. 1. Thus, biotinylated disks can be saturated with avidin by specific adsorption of the protein through one or more of its four binding sites and then allowed to take up biotin from an unknown test sample before being exposed to excess of radioactive biotin in order to determine the fraction of biotin sites on filter-bound avidin remaining unoccupied. The rationale for this sequential competition assay was the extremely low dissociation constant (*ca.* 10^{-15} *M*) of the avidin–biotin complex[14]. Thus, for all practical purposes, avidin-bound biotin will not exchange with free biotin in solution. Simple modification of the method allowed it to be used for the determination of avidin in unknown test solutions[13].

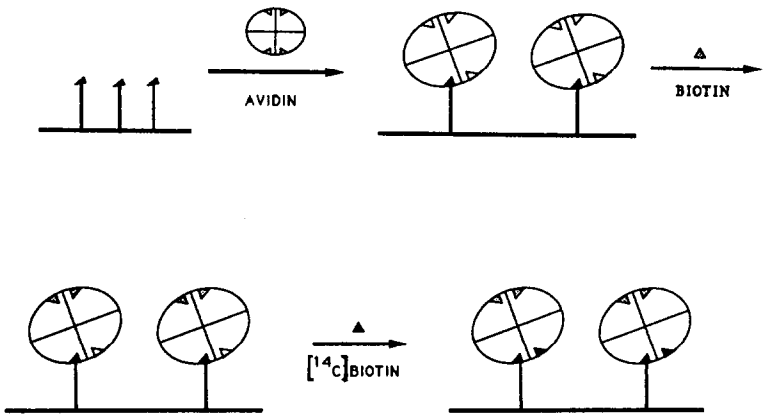


Fig. 1. Sequential competition assay for biotin.

The major limitation of he method as originally described was its narrow linear assay range. However, attempts to extend the useful range of the assay by increasing the density of covalently bound biotin in order to raise the avidin capacity of the disks did not produce the hoped for result. Although the amount of avidin adsorbed per disk did indeed increase as the density of matrix biotin was raised, this was not paralleled by a corresponding rise in the ability of the disks to take up free biotin from solution. On the contrary, in some instances a drastic decrease in biotin-binding capacity was observed at very high surface-bound biotin densities. This paper reports a systematic investigation of the deterioration phenomenon and proposes an explanation that probably has more general validity. The proposed model also suggested a practical approach to the problem of designing disks with wider linear ranges for biotin and avidin.

EXPERIMENTAL

Materials

Chromatographically purified avidin (10–15 U/mg protein), bovine serum albumin (BSA, fraction V), egg-white lysozyme and *d*-biotin were obtained from Sigma (St. Louis, MO, U.S.A.). *d*-Carbonyl[^{14}C]biotin (50 mCi/mmol) was purchased from the Radiochemical Centre (Amersham, U.K.). Potassium *tert.*-butoxide, obtained from Fluka (Buchs, Switzerland), was stored in small batches in hermetically sealed vials in a desiccator and used only once. 1-Tosyloxy-3-isocyanopropane was purchased form Fluka. All common reagents were of the purest grade available.

Disks of 12 mm diameter were cut from sheets of Whatman Grade 542 filter-paper (ashless, hardened) (Whatman, Maidstone, U.K.). Anhydrous dimethyl sulphoxide (DMSO) (Merck, Darmstadt, F.R.G.) was routinely treated with molecular sieve before use. Acetaldehyde was redistilled and stored in full tightly stoppered bottles at −20°C for up to 2 months.

Polyacrylation of cellulose disks

Polyacrylamide side-chains were grafted onto cellulose filter-paper disks by a modification of the procedures of Mino and Kaizerman[15] and Müller[16]. Untreated disks (7 g; about 1000 12-mm disks) were placed in a three-necked, round-bottomed flask connected to a mechanical shaker. An aqueous solution of acrylamide (3–6 g in 240 ml of water) was then added and the suspension was exhaustively deaerated under a water pump and then flushed with nitrogen. Graft polymerization was initiated by the addition of stock cerium(IV) ammonium nitrate solution (0.1 *M*) to a final initiator concentration of $4 \cdot 10^{-3}$ *M* (Type I disks) or $4 \cdot 10^{-5}$ *M* (Type II) disks (Table I). The polymerization reaction was allowed to proceed for 2 h at room temperature under nitrogen with gentle mechanical shaking. The grafted disks were then exhaustively washed with water and taken for hydroxymethylation. The nitrogen content of such disks was determined by the Kjeldahl method[17].

Hydroxymethylation of polyacrylamide side-chains on grafted disks

Hydroxymethyl groups were introduced on the polyacrylamide side-chains of the grafted disks essentially as described by Amarant and Bohak[18]. Washed, grafted disks (7 g) were suspended in an aqueous solution (200 ml) 5.2 *M* in formaldehyde and 0.05 *M* in sodium carbonate and allowed to react for 2 h at 50°C in a 250-ml

TABLE I

BASIC TYPES OF POLYACRYLAMIDE–CELLULOSE DISKS

Parameter	*Type I disks*			*Type II disks*		
Batch no.	1	2	3	4	5	6
Ce^{IV} concentration (*M*)		$4 \cdot 10^{-3}$			$4 \cdot 10^{-5}$	
Acrylamide monomer concentration (%)	1.25	2.0	2.5	1.25	2.5	5.0
Acrylamide content (μmol per disk)	5.5	7.5	10.8	5.6	7.7	8.9

stoppered flask with mechanical shaking. The disks were then exhaustively washed with water, methanol and diethyl ether and air dried. The dry disks were stored in a desiccator over phosphorus pentoxide.

Introduction of isonitrile functional groups

Isonitrile (–NC) functional groups were introduced on cellulose and hydroxymethylated polyacrylamide–cellulose disks as described previously[12,13,19–21]. Appropriate disks (7 g; about 1000 disks) were suspended in 85 ml of DMSO, allowed to swell with gentle mechanical shaking for 30 min in a stoppered flask and then brought to a final potassium *tert.*-butoxide concentration of 0.005–0.05 *M* by slow addition of 50–500 mg of the compound. Ionization of hydroxyl groups was allowed to proceed to equilibrium at 40°C for 15 min before addition of 1-tosyloxy-3-isocyanopropane (1.3 g, 0.006 mol) dissolved in DMSO (2.5 ml). The malodorous reaction was allowed to proceed for 4 h at 40°C with gentle mechanical shaking in a hood and the disks were removed, placed in a funnel, washed once with DMSO, several times with methanol, then diethyl ether and air dried. The disks could be stored indefinitely in closed vials at 4°C.

Biotinylation

Isonitrile disks (7 g) were suspended in 80 ml of ice-cold 0.1 *M* Tris buffer (pH 7) containing 50 mg of *d*-biotin in a tightly stoppered vessel. Cold, redistilled acetaldehyde (0.35 ml) was then added and the reaction was allowed to continue at 4°C for 18–24 h with gentle mechanical shaking. The disks were removed to a new vessel and then continuously flushed with tap water for 24 h in order to remove all traces of unbound biotin. The disks were sequentially washed with deionized water, methanol and diethyl ether on a suction funnel and air dried. Disks stored in the dry state are stable indefinitely, even at room temperature.

Avidinylation of biotinylated disks

Saturative avidinylation for biotin assay purposes was carried out with either commercial, chromatographically purified egg-white avidin or diluted egg-white as the avidin source[13], as follows.

About 20 disks were immersed for 3–4 h at 37°C in 10 ml of a solution containing 40–80 $\mu g\ ml^{-1}$ purified tetrameric avidin in 0.5 *M* potassium phosphate buffer (pH 7) made 0.005 *M* in cetyltrimethylammonium bromide (CTAB) and 0.01 *M* sodium azide. The mixture of polyvalent anion plus cationic detergent minimizes non-specific adsorption of avidin (a glycoprotein) to the cellulosic matrix of the disk.

Egg-white from one fresh egg (*ca.* 30 ml) was slowly mixed with one volume of 2% (v/v) Triton X-100 while magnetically stirring at room temperature. The resulting homogenate was then diluted 1:1 with 0.5 *M* potassium phosphate buffer (pH 7) made 5 m*M* in CTAB and 0.01 *M* in sodium azide and the gelatinous precipitate formed was removed by centrifugation at 8000 *g* and room temperature for 20 min. The clear supernatant (*ca.* 120 ml) was used without further treatment as a source of avidin tetramer. Depending on the disk capacity, overnight exposure to 1–2 ml of this solution at room temperature sufficed for saturative avidinylation. Avidin exposures of longer than 24 h are to be avoided for reasons that will become evident under Results and Discussion. Avidinylated disks were exhaustively washed with water prior to use.

Sequential competition assay for biotin[12,13]

The maximum capacity of a given batch of avidinylated disks for biotin was determined by immersion (for 30 min) in excess of [^{14}C]biotin (100 ng/ml per disk) followed by extensive washing with water, drying and counting in a β-scintillation spectrometer. Additional disks of the same batch were immersed in unknown biotin solutions (or dilutions thereof) for 3 h (five disks per 5-ml sample) and then washed extensively with water. The residual biotin-binding capacity of disks previously exposed to unknown biotin solution was determined by saturative exposure to [^{14}C]biotin as above. Subtraction of this residual value from the originally determined maximum disk capacity directly measured the amount of biotin in a sample (see Results and Discussion). Given the unusually high affinity of avidin for biotin ($K_D = 10^{-15}$ *M*)[14], there is essentially no exchange of unlabelled biotin with ^{14}C-labelled ligand.

Avidin assay[13]

The ability of biotinylated disks to remove avidin from crude mixtures forms the basis of a simple, quantitative assay for avidin.

Pretreatment of disks and glassware. Biotinylated disks were immersed in a solution containing 4 mg/ml each of lysozyme and BSA in PTA buffer [0.5 *M* potassium phosphate–1% (v/v) Triton X-100–0.01 *M* sodium azide, pH 7] for 10–15 min in order to minimize subsequent non-specific adsorption of avidin and other glycoproteins. Similarly, presoaking of glassware, micropipette tips and plastic microplates in a five-fold diluted solution of detergent-treated egg-white (see above) prevented loss of sample avidin to the walls of these containers. Quantitative measurements of standard avidin solutions are not obtained when these pretreatments are omitted.

Uptake of avidin from solution by biotinylated disks. Biotinylated disks pretreated as described above were placed, one disk per well, in the wells of a tissue-culture microplate of appropriate size. Avidin-containing test solutions were diluted 1:1 with double-strength PTA buffer and 200 μl of diluate were then placed on each disk. The plates were covered, incubated at 37°C for 3 h, washed successively with PTA buffer and water and then immersed in a standard solution of radioactive biotin to saturate the biotin-binding sites of avidin molecules taken up from the test solution. Based on results with purified avidin solutions, the relationship between the amount of radioactivity bound to disks and the avidin concentration in the test samples was linear.

Determination of –NC functional group content of isonitrile–cellulose and isonitrile–polyacrylamide–cellulose disks.

The –NC content of disks was estimated from the amount of [^{35}S]methionine bound by a four-component condensation (4CC) reaction[19,21]. Sets of five disks were suspended in 10 ml of 2 mM [^{35}S]methionine (specific radioactivity *ca.* 10^6 dpm μmol^{-1}) in 0.1 *M* potassium phosphate–0.5 *M* potassium acetate (pH 8) in a stoppered vial. Ice-cold acetaldehyde was added to a final concentration of 0.45 *M* (25 μl ml^{-1}) and the reaction mixture was shaken gently overnight at 4°C. Disks were removed, washed exhaustively with water and then successively washed with methanol and diethyl ether. The disks were air-dried and counted in a β-scintillation spectrometer at ^{35}S settings.

Direct determination of disk-bound avidin tetramer (A_4).

The avidin content of diluted egg-white solution was determined as described and then radioactively labelled with an equimolar amount of [^{14}C]biotin. Exposure of biotinylated disks to the labelled avidin solution made it possible to determine directly the precise amount of avidin tetramer bound to a disk.

RESULTS AND DISCUSSION

Preparation of biotinylated disks

The two basic types of functionalized disks used were isonitrile–cellulose, in which –NC functional groups were attached to the cellulose matrix itself, and isonitrile–polyacrylamide–cellulose, in which the –NC functional groups were attached to polyacrylamide side-chains grafted on the cellulosic matrix.

Polyacrylation of cellulose disks. Cellulose disks bearing polyacrylamide side-chains were prepared by graft polymerization of acrylamide according to a procedure[16] based on that of Mino and Kaizerman[15] for materials carrying surface primary or secondary alcoholic groups. According to the proposed mechanism, graft polymerization is started by a radical formed on the α-carbon of the hydroxylic function by the action of Ce^{IV} ion (reduced to Ce^{III} ion). The surface density of initiated polyacrylamide chains would hence depend on the concentration of Ce^{IV} ion.

Fig. 2 shows that the nitrogen content of grafted disks increases monotonously with increasing acrylamide monomer concentration (at [Ce^{IV}] = 10^{-3} M) and approaches asymptotically a limiting value of about 22 μmol per disk. In addition, the nitrogen content of the disks was found to be relatively insensitive to the concentration of Ce^{IV} in the range 10^{-3}–10^{-5} M (Table I). The concentration of the polymerization initiator therefore appears to control the surface density of polyacrylamide chains, but not the amount of acrylamide incorporated. This finding suggests that the Type II disks defined in Table I (*i.e.*, those prepared in the presence of 10^{-5} M Ce^{IV}) will have fewer, but longer, polyacrylamide side-chains than Type I disks (prepared in the presence of 10^{-3} *M* Ce^{IV}).

Introduction of isonitrile functional groups on cellulose and polyacrylamide–cellulose disks. The procedure used to introduce isonitrile functional groups on hydroxylic polymeric supports (*e.g.*, cellulose and other polysaccharides) has been described

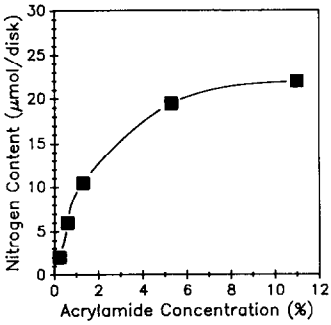


Fig. 2. Nitrogen content of polyacrylamide–cellulose disks as a function of acrylamide monomer concentration (Ce^{IV} ion concentration 4 × 10^{-3} *M*).

elsewhere[12,13,19,21]. In essence, it involves partial ionization of hydroxylic groups on the support with a strong base in a polar aprotic solvent (potassium *tert.*-butoxide in DMSO) followed by nucleophilic attack of the polymeric alkoxide ions on an isonitrile containing a good leaving group in the ω-position, *viz.*, 1-tosyloxy-3-isocyanopropane (p-$CH_3C_6H_4SO_2O(CH_2)_3NC$):

$$\vdash OH \xrightarrow[DMSO]{tert.\text{-}BuO^-} \vdash O^- \xrightarrow[DMSO,\ 30°C]{Tos\cdot O\cdot(CH_2)_3\cdot NC} \vdash O(CH_2)_3NC \qquad (1)$$

$$Tos = p\text{-}CH_3C_6H_4SO_2\text{-}$$

(where Bu = C_4H_9).

The procedure outlined in eqn. 1 could also be used to introduce –NC functional groups onto polyacrylamide side-chains of polyacrylamide–cellulose disks following partial hydroxymethylation of side-chain amide groups by treatment with concentrated formaldehyde at alkaline pH[18,20]:

$$\vdash CONH_2 \xrightarrow[pH\ 9.5]{HCHO} \vdash CONHCH_2OH \qquad (2)$$

$$\vdash CONHCH_2OH \xrightarrow[TosO(CH_2)_3NC]{tert.\text{-}BuO^-/DMSO} \vdash CONHCH_2O(CH_2)_3NC \qquad (3)$$

Fig. 3 shows that the –NC content of both cellulose and hydroxymethylated polyacrylamide–cellulose disks is a linear function of *tert.*-butoxide concentration. The linear dependence on base concentration allowed considerable flexibility in preparing disks having a wide range of functional group densities.

Biotinylation of functionalized disks. Biotin was covalently attached to functionalized disks by a 4CC reaction carried out in an aqueous buffer at neutral pH[19–21]. As shown in eqns. 4 and 5, 4CC reactions involve the simultaneous participation of amine, carboxylate, aldehyde and isonitrile, and lead to the formation of a stable N-substituted peptide bond between the carboxylate (R^1COOH) and amine (R^2NH_2)

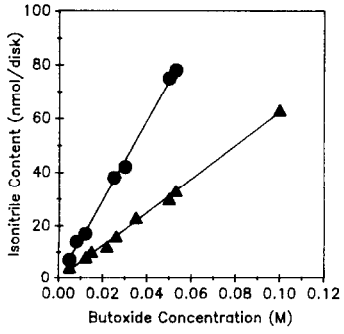
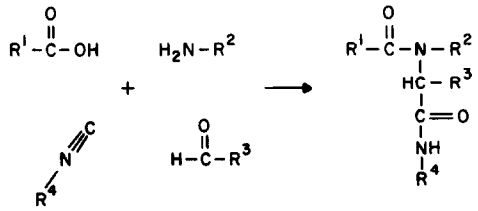


Fig. 3. Dependence of the isonitrile content of cellulose (▲) and polyacrylamide–cellulose disks (●) on the concentration of potassium *tert.*-butoxide, at a constant concentration of 1-tosyloxy-3-isocyanopropane (0.05 *M*).

moieties in which the aldehyde and the isonitrile components (R^3CHO and R^4NC) appear as the side-chain attached to the amide nitrogen. In our case, R^1COOH is biotin, NH_2 groups are supplied by Tris buffer, the carbonyl component is acetaldehyde and R^4NC is the functionalized cellulose or polyacrylamide–cellulose support.



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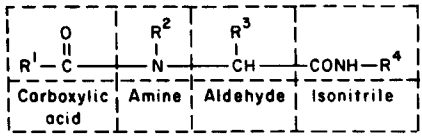


(5)

Representative data relating some properties of biotinylcellulose and biotinylpolyacrylamide–cellulose disks to functional group density are presented in Tables II and III (the data are discussed below).

Properties of biotinylcellulose disks

Fig. 4 summarizes the avidin- and biotin-binding properties of biotinylcellulose disks of varying functional group density (see also Table II). As far as their ability to bind avidin was concerned, such disks exhibited simple Langmuir-type saturation behaviour with increasing –NC group density. On the other hand, a biphasic response

TABLE II

BASIC CHARACTERISTICS OF BIOTINYLATED CELLULOSE DISKS

Batch No.	*–NC content*[a] *(nmol per disk)*	*Binding capacity*		*B/A_4 (mol/mol)*	*Disk designation*
		Avidin (μg per disk)	*[^{14}C]Biotin (ng per disk)*		
1	1.5	1.1	12	3	"High"
2	2.3	1.6	17	3	efficiency
3	4.0	2.6	29	3	
4	5.0	3.4	36	3	
5	6.1	4.0	44	3	
6	7.7	3.9	42	2.9	
7	9.1	4.0	43	3	
8	11.3	3.8	43	2.9	
9	13.8	3.7	38	2.7	"Low"
10	20.4	3.9	36	2.4	efficiency
11	40.0	4.0	26	1.8	
12	65.0	4.0	23	1.6	
13	100.0	3.9	22	1.6	

[a] Calculated from the amount of [^{35}S]methionine bound (see Experimental).

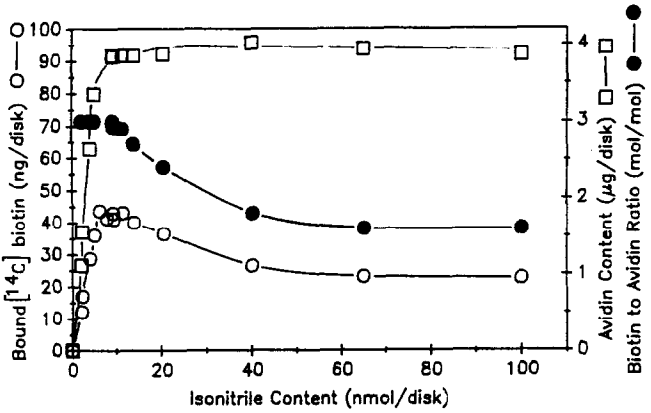


Fig. 4. Effect of functional group density on the subsequent ability of biotinylcellulose disks to bind avidin and radioactive biotin. ○ = [^{14}C]Biotin-binding capacity; □ = bound avidin content; ● = Biotin to tetrameric avidin ratio (B/A_4).

was observed when biotinylcellulose disks previously exposed to saturating amounts of avidin were challenged to radioactive biotin. The intitial rise in [^{14}C]biotin-binding capacity observed as the –NC group density increased to about 10 nmol per disk was followed by a monotonous decrease in biotin-binding capacity with a further increase in –NC density. This anomaly is emphasized when calculated molar ratios of biotin to avidin (B/A_4) are examined. Up to a critical –NC value (*ca.* 10 nmol per disk), B/A_4 assumes the theoretical value of 3 (reflecting the attachment of individual avidin tetramers to biotinylated disks via a single biotin-binding site). Beyond the critical inflection point, however, the apparent B/A_4 values decline asymptotically toward a value of *ca.* 1.5. Two types of biotinylcellulose disks could be defined operationally by the data in Fig. 4 and Table II: "high" efficiency disks ($B/A_4 \approx 3$) with functional group densities not exceeding 10 nmol per disk and "low" efficiency (*i.e.* high –NC group density) disks characterized by low biotin-binding capacities and sub-optimum (<3) B/A_4 ratios.

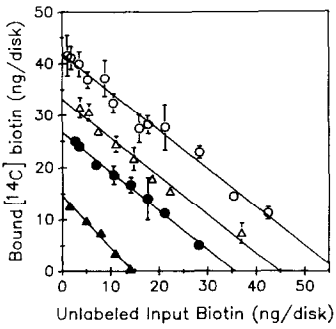


Fig. 5. Biotin assay ranges of avidinylated biotinylcellulose disks with diverse functional group densities. Isonitrile and avidin contents of tested batches of disks were (per disk): (▲) NC = 2.1 nmol, A_4 = 1.12 μg; (●) NC = 4.0 nmol, A_4 = 2.64 μg; (△) NC = 5.0 nmol, A_4 = 3.35 μg; (○) NC = 11.3 nmol, A_4 = 3.9 μg.

TABLE III

BASIC CHARACTERISTICS OF BIOTINYLATED POLYACRYLAMIDE–CELLULOSE DISKS

Disk Type[a]	*Batch No.*	*Acrylamide content (μmol per disk)*	*– NC content (nmol per disk)*	*–NC/AAm*[b]	*Binding capacity*		*B/A*$_4$ *(mol/mol)*
					Avidin (μg per disk)	*[^{14}C]Biotin (ng per disk)*	
Type I	1	5.5	3.9	0.0007	2.4	7.5	0.9
			13.3	0.0020	–	17	–
			42.5	0.0077	1.5	20	0.9
			76.7	0.0140	6.0	24	1.1
	2	7.5	10.2	0.0140	6.0	17	0.3
	3	10.8	7.1	0.0007	2.9	10	1.0
			15.8	0.0015	4.0	20	1.4
			40.0	0.0037	5.7	34	1.6
			48.0	0.0045	5.7	32	1.5
			300	0.0280	6.6	5.0	0.8
Type II	4	5.6	52.0	0.0090	8.2	90	3.0
	5	7.7	1.9	0.0003	0.7	7.2	3.0
			23.0	0.0030	4.5	46	3.0
			38.0	0.0050	7.3	63	2.8
			53.5	0.0070	7.7	77	2.7
	6	8.9	58.0	0.0065	8.7	95	3.0

[a] See Table I.

[b] –NC/AAm = side-chain isonitrile to acrylamide molar ratio.

Fig. 5 shows biotin assay ranges of "high"-efficiency biotinylcellulose disks of varying functional group content. As detailed under Experimental, sequential competition assay[12,13,22] for biotin consists of saturative avidinylation of the disks followed by their serial exposure to unknown or standard biotin solution and enough [^{14}C]biotin to ensure occupation of all remaining biotin sites on bound avidin molecules. The data show that every batch of disks examined gave a linear biotin-binding response whose range was a simple function of disk –NC density. As biotinylated disks remove avidin from solution, the batches of "high"-efficiency biotinylcellulose disks described above could also be used for the determination of avidin. As indicated in Fig. 6, it was always possible to obtain a range of avidin concentration over which the relationship between the amount of avidin added and final amount of radioactive biotin bound was linear (see Experimental for details). Moreover, the slopes of the linear regression lines so obtained were all identical. The avidin assay range of these disks was nonetheless limited as there was a tendency to depart from linear behaviour at avidin inputs below the saturation binding value imposed by their respective functional group densities (see also ref. 22). These limitations to the assay range prompted us to construct the grafted disks explored below.

Properties of Type I biotinylpolyacrylamide–cellulose disks

Fig. 7 indicates that Type I biotinylpolyacrylamide–cellulose disks (Table III) resemble biotinylcellulose disks in certain of their properties. Specifically, their avidin- and free biotin-binding capacities exhibit hyperbolic saturation behaviour and a biphasic response in relation to disk functional groups density, respectively. However, the avidin saturation value of these disks is roughly 1.5 times higher than that of the best biotinylcellulose disks obtained and, further, the inflection point of the [^{14}C]biotin-binding curve after saturative avidinylation occurs at a disk –NC content of *ca.* 40 nmol rather than the 10 nmol characteristic of biotinylcellulose disks.

Another striking property of Type I biotinylpolyacrylamide–cellulose disks evident from examination of Fig. 7 is their generally low apparent B/A_4 value. At even the optimum –NC functional group density this value never reaches 2, and falls well below 1 as the –NC content is raised even higher (see also Table III). In other words, disks with high enough functional group densities in effect become inactive. Thus, as indicated by the biotin and avidin assay curves in Figs. 8 and 9, the ligand-binding

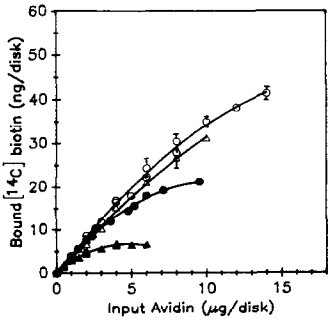


Fig. 6. Avidin assay ranges of biotinylcellulose disks. Batches of disks as in Fig. 5.

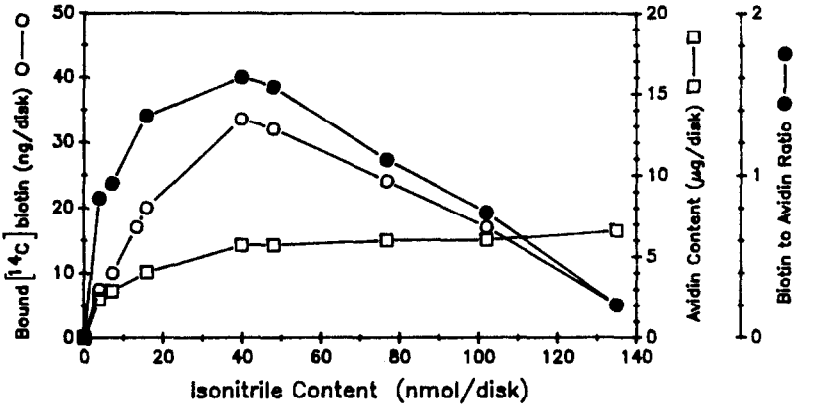
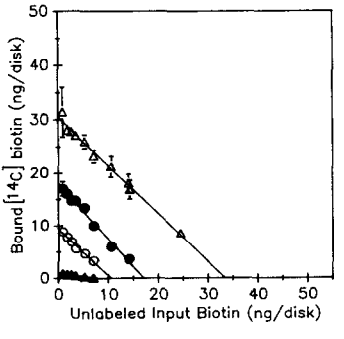


Fig. 7. Effect of functional group density on the subsequent ability of Type I biotinylpolyacrylamide–cellulose disks to bind avidin and radioactive biotin. (○) [^{14}C]Biotin binding capacity; (□) = bound avidin content; (●) = biotin to tetrameric avidin ratio. Details of disk preparation are presented under Experimental and in Tables I and III.

capacity progressively increases with increasing disk –NC content up to the inflection point (Fig. 7), and then declines nearly to zero. Also note the extended linear avidin assay range of the Type I polyacrylamide–cellulose disks in Fig. 9 compared with that of the cellulose disks in Fig. 6, in addition to the dependence of the slopes of the avidin response curves on disk –NC content. "High"-efficiency biotinylcellulose disks show no such dependence in the linear portions of their response curves.

Properties of Type II biotinylpolyacrylamide–cellulose disks

It will be recalled that the acrylamide contents of Type I and Type II polyacrylamide–cellulose disks were very similar (Table I and Fig. 2). As the concentration of polymerization initiator (Ce^{IV} ion) used to start the growth of polyacrylamide side-



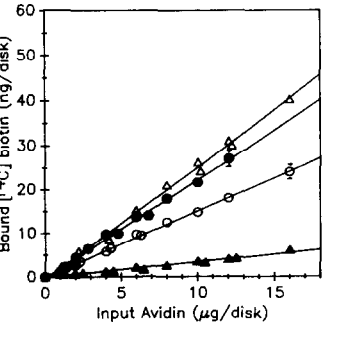


Fig. 8. Biotin assay ranges of avidinylated Type I biotinylpolyacrylamide–cellulose disks with diverse functional group densities. Isonitrile and avidin contents of tested batches of disks were (per disk): (○) NC = 7.1 nmol, A_4 = 2.9 μg; (●) NC = 15.8 nmol, A_4 = 4.0 μg; (△) NC = 48.0 nmol, A_4 = 5.7 μg; (4) (▲) NC = 301 nmol, A_4 = 6.6 μg (see also Table III).

Fig. 9. Avidin assay ranges of Type I biotinylpolyacrylamide–cellulose disks. Batches of disks as in Fig. 8.

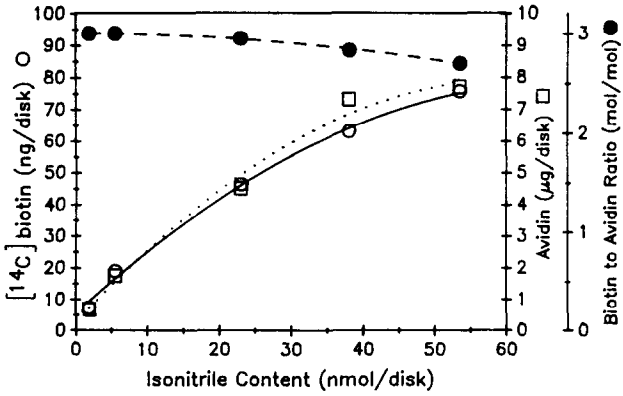


Fig. 10. Effect of functional group density on the subsequent ability of Type II biotinylpolyacrylamide–cellulose disks to bind avidin and radioactive biotin. (○) [^{14}C]Biotin binding capacity; (□) bound avidin content; (●) biotin to tetrameric avidin ratio. Details of disk preparation are presented in under Experimental and in Table III.

chains on Type II disks was 100-fold lower than in the case of Type I disks, it is reasonable to suppose that Type II disks possessed a smaller number of longer polyacrylamide side-chains compared with disks of Type I. The functional consequences of this difference are indicated by the avidin- and biotin-binding curves in Fig. 10. Thus, disks of about 50 nmol –NC content bound *ca.* 7 μg of avidin and were capable of ligating *ca.* 80 ng of free biotin after saturative avidinylation, compared with *ca.* 30 ng for similar Type I disks (see Table III). In other words, while similar to Type I disks with regard to avidin-binding capacity, Type II disks appeared to possess a much higher capacity for free biotin. In addition, their apparent B/A_4 values did not deviate much from the theoretical value of 3 over the entire –NC range tested. In comparison, the best B/A_4 value for Type I disks (50 nmol –NC content) was only 1.7 and was very dependent on –NC content (Fig. 7 and Table III). Moreover, as Fig. 11 shows, the linear range of standard avidin assay curves obtained with different batches of Type II disks was as extended as that observed with Type I disks (Fig. 9), while the slopes of these curves were independent of –NC content and similar in magnitude

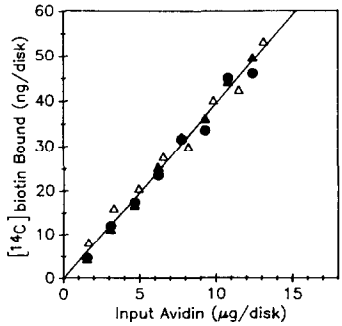


Fig. 11. Avidin assay ranges of Type II biotinylpolyacrylamide–cellulose disks. The three batches of disks used respectively contained (▲) 5.5, (●) 38 and (△) 55 nmol of isonitrile groups per disk.

to those for "high"-efficiency cellulose disks (Fig. 6). Accordingly, from a practical point of view, Type II polyacrylamide–cellulose disks become the vehicles of choice for avidin and biotin assay as they combine the extended linearity of Type I polyacrylamide–cellulose disks with the enhanced sensitivity of "high"-efficiency cellulose disks.

Time dependence of avidin and biotin assays with biotinylcellulose disks

As shown schematically in Fig. 12, differences in biotinylated disk behaviour can be explained by assuming both single- and multi-site attachment of avidin tetramers to cellulose (Fig. 12A) and polyacrylamide–cellulose (Fig. 12B and C) disks. Thus, the probability of multi-site avidin attachment to more than one polyacrylamide side-chain in a polyacrylamide–cellulose disk would be low when such chains are widely spaced (as in the case of Type II disks), and increase in frequency with increasing side-chain density (*i.e.*, Type I disks). In other words, the observed B/A_4 ratios of 3 in Type II polyacrylamide–cellulose disks and <3 in Type I polyacrylamide–cellulose disks are in keeping with the predictions of the above model. The same basic model also explains the behaviour of "high"- and "low"-efficiency cellulose disks, including the fact that the B/A_4 values of "high"-efficiency cellulose disks attain the limiting value of 3.

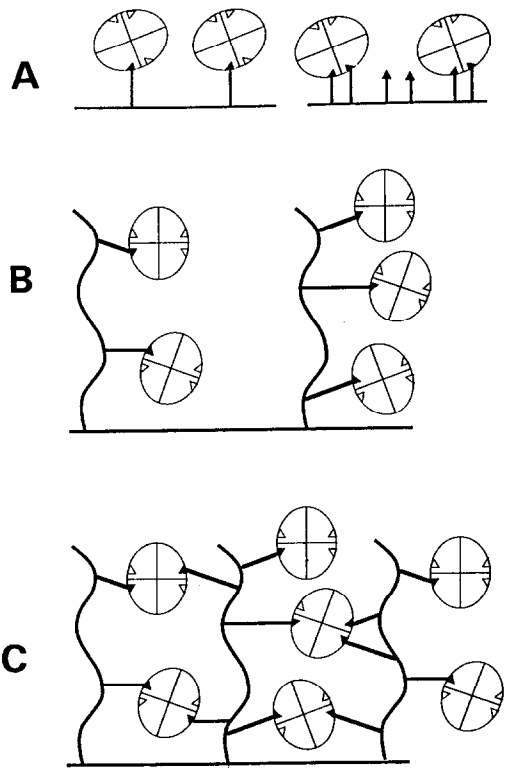


Fig. 12. Single- and multi-site attachment of avidin to (A) biotinylated cellulose and (B and C) polyacrylamide–cellulose disks.

One of the undesirable characteristics of "low"-efficiency avidinylated biotinylcellulose disks was their irreproducibility as biotin assay vehicles. Depending on the time and conditions of pre-exposure to avidin, the biotin-binding capacity of such disks varied from relatively good to almost zero. This observation can also be accommodated by the model described above by assuming that time-dependent changes from single- to multi-site avidin attachment can occur. Experimental support for this assumption comes from the kinetic data summarized in Fig. 13. The indicated curves represent parallel experiments with "high"-(triangles) and "low"-efficiency (circles) biotinylcellulose disks. They demonstrate time-dependent changes in the amount of avidin bound (A), in subsequent saturative [^{14}C]biotin binding (B) and in calculated B/A_4 ratios (C) as a function of time of exposure to excess of avidin. As can be seen,

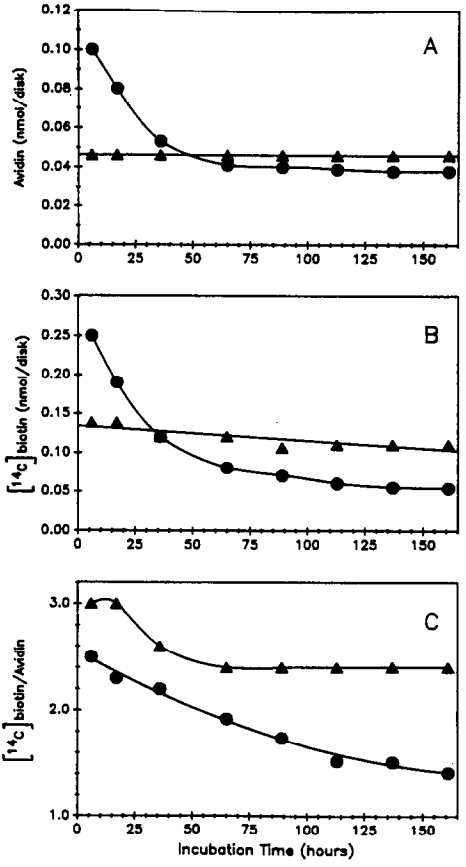


Fig. 13. Decay of disk-bound avidin function with time. Biotinylcellulose disks of low and high functional group density were derived from batches of disks averaging (▲) 7.6 and (●) 108 nmol of isonitrile groups per disk, respectively. Disks were immersed in an excess of ^{14}C-labelled avidin at 37°C for the indicated times. Some of the multiple number of disks removed at each time point were directly subjected to radioactivity determination in order to obtain their bound avidin content (A), whereas others were further exposed to excess of [^{14}C]biotin before counting in order to determine the total biotin binding capacity of each disk (B). The experimental values obtained were then used to calculate the number of biotin-binding sites per avidin (A_4) molecule (C).

the avidin-binding capacity of "high"-efficiency disks was constant with respect to time, the [^{14}C]biotin capacity of such disks was almost independent of time and the B/A_4 ratio varied from 3 at exposure times under 30 h to about 2.6 at longer exposure times. Presumably, disks with even lower functional group densities would have exhibited a time-independent B/A_4 value of 3. By contrast, "low"-efficiency disks initially showed a relatively high capacity for avidin, which declined steeply with time and reached a final plateau value lower than that of "high"-efficiency disks. "Low"-efficiency disks initially bound more free biotin than their "high"-efficiency counterparts, but lost biotin-binding capacity with time and approached a value only half that of the "high"-efficiency disks. Thus, the initial B/A_4 values of the "low"-efficiency disks were no higher than 2.5 and, in contrast to those of "high"-efficiency disks, steadily declined with time and approached a value of about 1.

Avidin molecules can be arranged in a two-dimensional array with either their long (5.5 nm) or short (4.1 nm) axis[14] lying perpendicular to a surface. In the former arrangement, each molecule of avidin occupies a smaller equivalent surface area (13.2 *versus* 23.8 nm^2) and hence permits a higher maximum molecular packing density at saturation. The only way to attain such maximum avidin packing is through single-site attachment (Fig. 12). Accordingly, the data for "low"- and "high"-efficiency cellulose disks in Fig. 13 can be interpreted in the light of the time-induced changes in avidin orientation depicted schematically in Fig. 14. Thus, the finding that the avidin content of "low"-efficiency disks decreased by a factor of about 2 with time can mean that half of the initially bound tetramers were forced off the disks by a shift from one to two-site attachement. Also, the fact that the B/A_4 ratio of "low"-efficiency disks continues to decrease with time even after disk avidin content has stabilized implies that the transition from one- to two-site attachment is followed by some degree of two- to three-site attachment (Fig. 14). The latter stage would not be expected to induce significant changes in the surface orientation of bound avidin molecules.

The "high"-efficiency cellulose disks used to obtain the kinetic data in Fig. 13 were similar in functional group density to batch number 8 disks in Table II and are thus borderline in behaviour to "low"-efficiency disks. These borderline disks departed from ideal "high"-efficiency behaviour in that they exhibited a small decrease in biotin-binding capacity with time. However, their bound avidin content remained constant with time and the B/A_4 ratio stabilized at a number close to the ideal value of 3. The findings suggest that most avidin molecules are attached via a single site in "high"-efficiency disks, and that few molecules can shift to a two-site binding mode with time owing to the relative dearth of surface-bound biotin. The fact that the avidin plateau is almost the same for both "high"- and "low"-efficiency cellulose disks (Fig. 13A) suggests that the avidin content of the former is as close to saturation as random orientation would allow.

The model outlined above on the basis of this study is probably general and helps to explain phenomena related to time- and concentration-associated activity

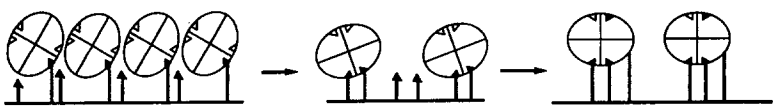


Fig. 14. Time-induced changes in the orientation of surface-bound avidin.

deterioration in affinity systems of high ligand density (see also refs. 23–25). For example, Hammond *et al.*[11] recently showed that affinity purification of recombinant protein A[26] by binding to immobilized IgG gave quantitative protein recoveries only up to a certain immobilized ligand density. At higher densities of column-bound IgG, the recovery of protein A declined almost to zero. Presumably, attachment of protein A to IgG is mainly of a single-site nature when bound IgG levels are low and progressively shifts to a stronger two-site mode of binding as the immobilized ligand content increases. Similarly, the degree of recovery of specific populations of functionally intact cells from immunoadsorbent affinity columns is known to be inversely related to the level of antibody substitution on the matrix[9,10]. Again, poor recovery can be explained by assuming that each cell-immobilized ligand complex will, on average, be more strongly held together by a greater number of contact points as the density of surface-bound ligands is raised.

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