Sepharose-Avidin Column for the Binding of Biotin or Biotin-Containing Peptides

The method of attaching a biologically active protein, e.g., an enzyme to an activated polysaccharide matrix[1] has become widely used in recent years, since on the inert surface the enzyme retains most of its activity and is easy to handle. Selective purification methods for proteins based on such affinity chromatography[2] were devised like the single step isolation-purification of avidin from egg white[3].

We found that avidin can be coupled to Sepharose 4B activated with cyanogen bromide. A column prepared of this Sepharose-avidin showed considerable biotin or dye[4] binding capacity. For binding of biotin or biotin containing peptides such columns are quite efficient, recovery of the biotin containing material may be less practical.

In a typical experiment 20 ml agarose gel (Sepharose 4B from Pharmacia) was suspended in 20 ml water and a solution of 2 g CNBr in 20 ml water was added. The pH was raised and maintained at about 11 by the dropwise addition of 4 N NaOH. When the pH did not fall any more (about 10 ml 4 N NaOH was needed) the suspension was filtered on Buchner funnel and washed with 0.1 M $NaHCO_3$. To this activated gel were added 20 mg of avidin (Worthington) in 2 ml 0.1 M $NaHCO_3$ and the mixture was stirred at 4 °C for 20 h. The Sepharose-avidin was then poured into a chromatography tube, washed with 0.1 M $NaHCO_3$, and finally with 0.2 M phosphate buffer of pH 6.8. The UV-absorption of the effluents was measured at 280 nm to find unbound avidin. At least 80% of the avidin was bound by the gel.

A sample of this Sepharose-avidin (1 ml after low speed centrifugation) turned pink when treated with a 100 μM solution of 4-hydroxyazobenzene-2′-carboxylic acid. It was washed with 3 ml of 0.2 M phosphate buffer, pH 6.8, and then titrated with a biotin solution (0.034 mg/ml) until the red color disappeared (0.45 ml biotin solution). Thus, the Sepharose-avidin has a biotin binding capacity of 0.06 μmol/ml or 15 γ/ml.

The Sepharose-avidin was used to bind biotin containing fragments from a tryptic digest of methylmalonyl-oxaloacetic transcarboxylase[5] containing tritiated biotin. The solution, 336,000 counts/min in 4.5 ml of 0.2 M phosphate buffer pH 6.8 was poured over a column prepared from 4 ml Sepharose-avidin and was washed with 0.2 M phosphate buffer of pH 6.8. The column was dyed red with 5 ml 100 μM 4-hydroxyazobenzene-2′-carboxylate in 0.2 M phosphate buffer of pH 6.8, and washed with 6 ml of the same buffer. According to previous titration this column can bind 0.24 μmol of biotin. When the transcarboxylase digest was poured over the red column, its top one-third was discolored. The column was then washed with 0.2 M phosphate buffer and fractions of 8–10 ml were collected and counted. A total of 900 counts/min was found in 4 fractions. Elution with a 6 M guanidine-hydrochloride-hydrochloric acid solution of pH 1.5[3] removed only a total of 80,000 counts/min, or about 24% of the biotin containing fragments.

Zusammenfassung. Avidin konnte an das Polysaccharid Sepharose 4B, das zuvor mit Bromcyan aktiviert wurde, gebunden werden. Um Biotin oder Biotin-haltige Peptide zu binden, erwies sich eine Sepharose-Avidin-Säule als besonders günstig.

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[1] R. AZEN, J. PORATH and S. ERNBACK, Nature *214*, 1302 (1967).
[2] P. CUATRECASAS, M. WILCHECK and C. B. ANFINSEN, Biochemistry *61*, 636 (1968).
[3] P. CUATRECASAS and M. WILCHEK, Biochem. biophys. Res. Commun. *33*, 235 (1968).
[4] N. M. GREEN, Biochem. J. *94*, 23c (1965).
[5] H. G. WOOD, S. H. G. ALLEN, R. STJERNHOLM and B. JACOBSON, J. biol. Chem. *238*, 547 (1963).

Fluorometric Detection of Serotonin Using o-Phthaldialdehyde: an Improvement

Reaction of *o*-phthaldialdehyde (OPT) with serotonin standards increases amine detection sensitivity[1] over the direct BOGDANSKI procedure[2]; however, no detailed information is available concerning the blank which is a consistent problem when low concentrations of serotonin are being analyzed. The blank arises primarily from reagents and glassware and is variable and not easily controllable[3]. In the BOGDANSKI procedure, if blank fluorescence is large relative to serotonin fluorescence, the peak of maximal serotonin fluorescence (545 nm) may not be present but appears in the spectrum at a shorter wavelength. This apparent shift (atypicallity) is due to overlap of blank (ca. 400 nm wavelength) and serotonin (545 nm wavelength) emissions which are detected additively. Readings from the atypical peak or at 545 nm wavelength are inaccurate. We have reported[3] that the blank in the serotonin-OPT method constitutes a greater problem than in the BOGDANSKI procedure since reagent OPT produces exaggerated primary scattering, the increased volume of HCl required to achieve optimal acidity[4] produces greater blank fluorescence, and the peak of maximal serotonin-OPT fluorescence occurs at 473 nm wavelength in comparison to the maximal emission of serotonin at 545 nm. Reported here are the advantages of a chloroform wash in the serotonin-OPT method; chloroform removes major blank emissions and reduces primary scattering.

[1] R. P. MAICKEL and F. P. MILLER, Analyt. Chem. *38*, 1937 (1966).
[2] D. F. BOGDANSKI, A. PLETSCHER, B. B. BRODIE and S. UDENFRIEND, J. Pharmac. *117*, 82 (1956).
[3] J. H. THOMPSON, C. A. SPEZIA and M. ANGULO, Biochem. Pharmac., submitted.
[4] J. H. THOMPSON, C. A. SPEZIA and M. ANGULO, Experientia *25*, 927 (1969).

Materials and methods. Fluorescence was recorded using a spectrophotofluorometer (SPF)[5]. Specimens were activated at 355 nm wavelength and spectra were recorded[6] from 200–800 nm wavelength. Serotonin-OPT fluorescence was read at 473 nm wavelength. Details of SPF slit sizes and serotonin, and reagent and tissue preparation have been presented previously[7–9].

Specimens are extracted from the *n*-butanol, *n*-heptane mixture[1] into 1.5 ml of 0.1*N* HCl. 1.0 ml aliquots are transferred to acid washed glass-stoppered test tubes. 0.2 ml of 0.05 g/100 ml (w./v.) OPT in absolute ethanol is added to each tube. After mixing, 2.0 ml of 10*N* HCl are added with mixing. Tubes are then flushed for 60 sec with a light jet of filtered, dried nitrogen gas and capped.

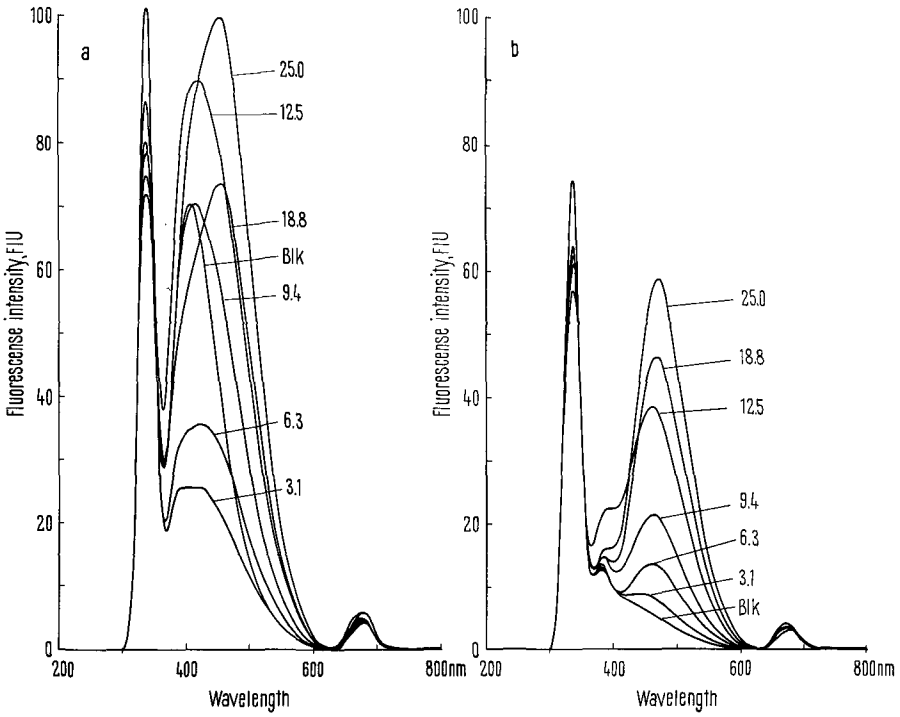

Fig. 1. Fluorescence spectra of blank and serotonin-OPT standards before (a) and after (b) a chloroform wash. Concentrations of the serotonin-OPT standards in final solution read were 25.0, 18.8, 12.5, 9.4, 6.3 and 3.1 ng/ml. Instrumental conditions were activation wavelength 355 nm, attenuation 1% full scale, sensitivity 0 sensitivity units and cell temperature 20 °C.

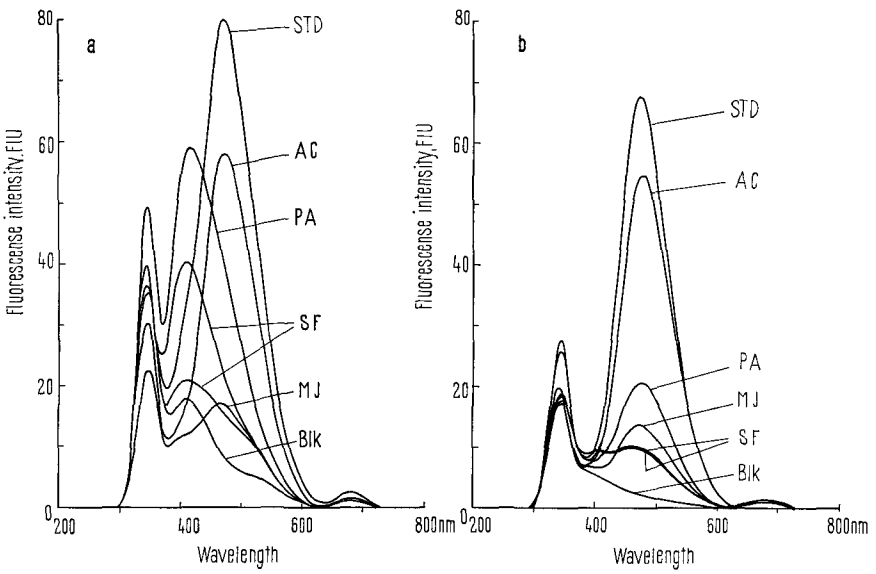

Fig. 2. Fluorescence spectra of extracted blank, serotonin-OPT standard and tissue specimens before (a) and after (b) a chloroform wash. Individual specimens were prepared by taking 0 and 1.2 µg of serotonin and various quantities of mucosa from a 200 g, male Sprague-Dawley rat. Tissues were stomach fundus in duplicate (SF), pyloric antrum (PA), mid-jejunum (MJ), and ascending colon (AC). The concentration of the serotonin-OPT standard in the final solution read was 61.5 ng/ml, instrumental conditions for all specimens were 355 nm, activation wavelength and 20 °C cell temperature. SF was read at an attenuation of 30% full scale and sensitivity of 60 sensitivity units. All other specimens were read at an attenuation of 10% full scale and a sensitivity of 40 sensitivity units.

Specimens are complexed with OPT in a 100 °C waterbath for 10 min. Tubes are cooled to room temperature in running tap water and read. Specimens extracted with chloroform are handled as follows: 3.2 ml of chloroform are added to each tube after OPT complexing and cooling to room temperature. Tubes are then shaken for 5 min using a platform shaker[10] and then centrifuged for 5 min at 2300 rpm[11] to aid partition. 1.5 ml of the upper, aqueous phase is taken for reading.

Results. Serotonin-OPT Standards. In the absence of a chloroform wash the blank produced atypicallity (Figure 1, a), and high and variable primary scattering. Specimens washed with chloroform, however, showed reduced primary scattering and blank peaks (Figure 1, b) with the production of characteristic serotonin-OPT spectra peaking maximally at 473 nm wavelength.

Tissues. Gross interference by blank components (Figure 2, a) producing atypical spectra is seen in the duplicates of SF, and in the PA; a small blank contribution is seen in the MJ sample producing minimal atypicallity. The AC peaks at 473 nm wavelength and although it contains a blank this component, relative to the serotonin-OPT fluorescence, is minimal and does not produce atypicallity. Specimens washed with chloroform (Figure 2, b) show reduced primary scattering and characteristic serotonin-OPT peaks; the duplicate stomach fundi are superimposable.

Serotonin-OPT Solubility. The partition isotherm[12] for serotonin-OPT at room temperature (20 °C) was linear for serotonin concentrations 40–200 ng/ml (Figure 3). The partition coefficient (K) was 4.9 ± 0.3 ($n = 5$). The chloroform wash removed 17% of the serotonin-OPT complex.

Discussion. Reagent blanks are frequently a problem in fluorometry especially when biological specimens are available in small quantities and their assay conducted in ppb. Use of high quality reagents and water has not solved this problem[2,3,13,14]. Filters cannot be used to selectively remove blank fluorescence at the wavelength of maximal serotonin or serotonin-OPT fluorescence. Therefore, they do not improve the accuracy of reading but in fact reduce detection sensitivity[3]. A single wash with an equal volume of chloroform was found to lower blank fluorescence with only a 17% loss of serotonin-OPT, and to yield typical serotonin-OPT spectra peaking at 473 nm wavelength. Reduced serotonin-OPT loss can be achieved by using a smaller chloroform: aqueous ratio. The chloroform wash was found to reduce and standardize primary scattering by removal of precipitated OPT, and to have no effect on acidity and temperature relationships for maximal fluorescence[4] or the excitation and emission spectra of serotonin-OPT (THOMPSON and SPEZIA, unpublished). Use of chloroform enables a greater detection sensitivity for serotonin-OPT[3]; it has been in use in our laboratory for over 2 years[15].

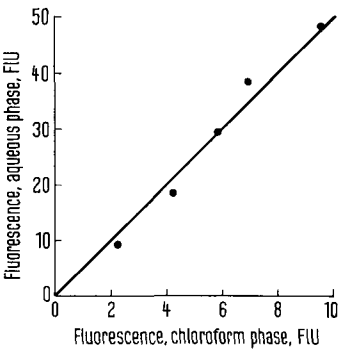

Fig. 3. Partition isotherm for serotonin-OPT in chloroform at 20 °C. Fluorescence intensity of aqueous phase obtained from spectra of 40, 80, 120, 160 and 200 ng/ml serotonin-OPT standards. Instrumental conditions were activation wavelength 355 nm, attenuation 0.3% full scale, sensitivity 0 sensitivity units and cell temperature 20 °C. Fluorescence intensity of chloroform phase determined by difference.

Résumé. Dans la détermination fluorimétrique du complexe sérotonine-OPT, le lavage des spécimens avec un volume égal de chloroforme, avant de l'évaluation, réduit l'interférence des blancs et la dispersion primaire. Cela permet une évaluation plus exacte de la fluorescence de la sérotonine et, dans quelques cas, l'usage de spectres autrement illisibles.

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[5] American Instrument Co., Inc., Aminco-Bowman, 8030 Georgia Avenue, Silver Spring (Maryland 20910, USA).
[6] Honeywell 320 Solid State XY Recorder, 4800 East Dry Creek Rd., Denver (Colorado 80217, USA).
[7] J. H. THOMPSON, C. A. SPEZIA and M. ANGULO, Experientia *25*, 1007 (1969).
[8] J. H. THOMPSON, C. A. SPEZIA and M. ANGULO, Analyt. Biochem., *31*, 321 (1969).
[9] J. H. THOMPSON, Ir. J. med. Sci. *490*, 411 (1966).
[10] Precision Scientific Co., 3737 W. Cortland St., Chicago, Ill. (USA Model 65885).
[11] International Equipment Co., 300 2nd Avenue, Needham Heights, Massachusetts 02194.
[12] L. C. CRAIG and D. CRAIG, in *Technique of Organic Chemistry* (Ed. A. WEISSBERGER; Interscience Publishers, Inc., New York 1950), p. 182.
[13] W. B. QUAY, Analyt. Biochem. *5*, 51 (1963).
[14] R. M. FLEMING, W. G. CLARK, E. D. FENSTER and J. C. TOWNE, Analyt. Chem. *37*, 692 (1965).
[15] Supported in part by the American Medical Association Education and Research Foundation.

Die näherungsweise graphische Darstellung von Isopotentiallinien aus EEG-Mehrkanalregistrierungen mittels EDV-Anlage

Bei Untersuchungen, in denen eine Variable (zum Beispiel bioelektrische Spannungen) eines zweidimensionalen Prozesses (oder eines dreidimensionalen, der nur an der Oberfläche abgeleitet wird) mittels mehrerer Elektroden registriert wird, erscheint es wünschenswert, eine zumindest annähernde Darstellung dieses zweidimensionalen Prozesses zu erhalten, da die Mehrkanalregistrierung recht unanschaulich und ihr zum Beispiel eine Verlagerung von Spannungshügeln kaum zu entnehmen ist. Eine Verbesserung ergibt sich durch die Topogrammdarstel-