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DERIVATIZATION OF COMPOUNDS AT THE ORIGIN OF THIN-LAYER PLATES WITH FLUORESCAMINE

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SUMMARY

A method has been developed for the detection of compounds with primary amino groups on thin-layer chromatography (TLC) plates with fluorescamine. The compounds dissolved in buffer were applied to TLC plates and derivatized at the origin by developing with or dipping into an acetone–hexane solution of fluorescamine. Virtually all fluorescamine derivatives stayed at the origin, and they were subsequently separated using appropriate solvent systems. As little as 10 pmoles of fluorescamine-derivatized compound could be detected when viewed under a long-wave (366 nm) ultraviolet lamp. The method was applied to the analysis of peptides, amino acids and amines.

INTRODUCTION

Fluorescamine has been widely used as a potent fluorogenic reagent for primary amino groups[1]. Adaptation of this versatile reagent to the detection of compounds on thin-layer chromatographic (TLC) plates has been reported by several authors[2–5]. Imai *et al.*[6] increased the sensitivity of detection by reacting the compounds with fluorescamine in test tubes before spotting. However, this prelabeling method has two shortcomings: dilution of the samples with reagents, and the need to react each sample individually. The present paper describes a new, more sensitive and convenient method involving the derivatization of buffered samples at the origin of TLC plates with fluorescamine and subsequent separation of the derivatives in the usual manner. Application of the method to biologically active peptides is also described.

EXPERIMENTAL

Reagent and solvents

The following were purchased from commercial sources: fluorescamine and somatostatin tetradecapeptide (H–Ala–Gly–Cys–Lys–Asn–Phe–Phe–Trp–Lys–Thr–Phe–Thr–Ser–Cys–OH) from Pierce (Rockford, Ill., U.S.A.); lysyl–bradykinin, methionyl–lysyl–bradykinin, (Asp[1] Ile[5]) angiotensin I, (Asp[1] Ile[5]) angiotensin II, (Arg[8]) vasopressin, angiotensin II hexapeptide (Val–Tyr–Ile–His–Pro–Phe), angio-

tensin II heptapeptide (Arg–Val–Tyr–Ile–His–Pro–Phe), tridecapeptide renin substrate (Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu–Leu–Val–Tyr–Ser) and tetradecapeptide renin substrate (Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu–Leu–Val–Tyr–Ser) from Schwartz/Mann (Orangeburg, N.Y., U.S.A.); caerulein (Asp–Tyr–Thr–Gly–Trp–Met–Asp–Phe–NH_2) and the heptapeptide corresponding to residues 21–27 of secretin (Arg–Leu–Leu–Gln–Gly–Leu–Val–NH_2) from Research Plus Labs. (Denville, N.J., U.S.A.); acetone, *n*-butanol, benzene, hexane, methanol, ethyl acetate and dioxane (all distilled-in-glass) from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.); petroleum ether, ethylene glycol monomethyl ether acetate, ethanol monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$) and dipotassium hydrogen phosphate (K_2HPO_4) from Fisher (Fair Lawn, N.J., U.S.A.). Other compounds were obtained from Sigma (St. Louis, Mo., U.S.A.).

TLC plates

The following glass chromatoplates (20 × 20 cm, without fluorescent indicator) were used without any treatment: precoated silica gel 60 TLC plate (0.25 mm thick) and precoated cellulose TLC plate (0.10 mm) from Merck (Darmstadt, G.F.R.); silica gel plates Q5 (0.25 mm) and LQ (0.25 mm, preadsorbent plate) from Quantum Industries (Fairfield, N.J., U.S.A.).

Preparation of stock solutions

Derivatization reagent was prepared by dissolving 10 mg of fluorescamine in 20 ml acetone and then adding 80 ml hexane. This solution is stable for at least one week. A 1-m*M* solution of penta-L-alanine was prepared in 0.2 *M* sodium borate buffer (pH 9.0)–ethanol (1:1). *n*-Heptylamine, *n*-octylamine and *β*-naphthylamine were dissolved first in the borate–ethanol solution to 5 m*M* and then diluted with 0.2 *M* sodium borate buffer (pH 9.0) to 1 m*M*. Proteins were dissolved in 0.2 *M* sodium borate buffer (pH 9.0) to 5 mg/ml. Catecholamines and amino sugars were dissolved in 0.2 *M* phosphate buffer (pH 8.0) to 1 m*M*. Other test compounds were dissolved in the borate buffer to 1 m*M*.

Solvent systems for separation of fluorescamine derivatives

The following solvent systems were used: (A) ethyl acetate–hexane–methanol–water (60:20:25:10), (B) chloroform–isopropanol–water (2:8:1), (C) acetone–ethyl acetate–methanol–water (3:2:1:1), (D) *n*-butanol–ethanol–methanol–water (6:4:2:1), and (E) dioxane–triethanolamine–methanol–water (6:1:1:1).

Fluorescamine derivatization of compounds at the origin of TLC plates

Predevelopment method. A 1-*μ*l sample was applied with a volumetric micropipette ("Microcaps"; Drummond, Broomall, Pa., U.S.A.) 1.5 cm from the lower edge of a TLC plate. After air drying, the plate was placed in a tank containing a 1 cm depth of derivatization reagent. After the solvent front had moved at least 10 cm, the plate was removed and the solvent was evaporated with a hairdryer. The plate was then developed with an appropriate solvent system. Fluorescence was observed under a long-wave (366 nm) ultraviolet lamp after air drying. Prelabeling and development of the plates were performed in tanks wrapped in aluminum foil to protect the samples from light.

Predipping method. The lower 2 cm of the spotted plate was dipped in the derivatizing reagent for 30 min, after which the plate was dried and developed as described above.

Determination of extent of the reaction of primary amino groups with fluorescamine on TLC plates

An aliquot (1 μl) of stock solution (1 m*M* or 5 mg/ml), equivalent to $1 \cdot 10^{-9}$ mole or 5 μg, was spotted on to a silica gel 60 plate and developed for the indicated distance or dipped for the indicated time in the derivatizing reagent. After air drying, the sample was scraped from the plate, transferred to a glass vial (Kimble; disposable scintillation vial), and extracted with 3.5 ml of a solution consisting of acetone–0.2 *M* sodium borate pH 9.0 buffer (1:2.5)[7]. After vigorous shaking for 10 sec on a vortex-type mixer, the fluorescence of the supernatant was measured in an Aminco-Bowman spectrofluorometer (excitation 390 nm, fluorescence 490 nm).

The extent of the reaction was determined by comparing the fluorescence with that obtained with samples reacted with fluorescamine before application to the TLC plate. To 50 μl of a 2-m*M* or 10-mg/ml solution of test compound was added 450 μl of fluorescamine (20 mg per 100 ml acetone) with vigorous mixing with a vortex mixer. A 5-μl aliquot (equivalent to $1 \cdot 10^{-9}$ mole or 5 μg) was applied to another silica gel 60 plate and treated as described above, except that fluorescamine was omitted from the acetone–hexane solution.

Recovery of fluorescamine derivatives from the TLC plate was calculated by comparison with the fluorescence intensity of 5 μl of reaction mixture added to 3.5 ml extracting solution. Values were corrected with the appropriate blanks.

RESULTS

Solvent for pre-derivatization of compounds on TLC plates

Derivatization of compounds with fluorescamine on TLC plates must be performed in such a manner that the derivatives are not displaced from the origin. Silica gel plates with a preadsorbent sample dispensing area (LQ plate; Quantum Industries) were tested with amines, amino acids and peptides and found unsatisfactory because no solvents for fluorescamine were found which permitted the concentration of derivatives at the junction of the two layers. Satisfactory results were obtained with silica gel 60 plates which permitted labeling at the origin without migration of the spots when the appropriate solvent for fluorescamine was used. The common solvents acetone and dioxane moved the derivatives of less polar compounds such as β-naphthylamine, *p*-aminobenzoic acid, L-Phe–L-Phe, alkylamines, tyramine, octopamine, tryptamine and DL-normetanephrine. Ethyl acetate, another good solvent for fluorescamine, also displaced some derivatives. Benzene, hexane and petroleum ether were poor solvents for fluorescamine but they displaced only the β-naphthylamine derivative. An acetone–hexane (1:4) solution was a suitable solvent for fluorescamine and displaced none of the compounds tested except β-naphthylamine. Although benzene and acetone–hexane (1:4) gave almost the same results, the latter was chosen because of its shorter developing time, faster reaction rate, higher solubility for fluorescamine, and lower toxicity.

Reactivity of compounds with fluorescamine on TLC plates

Reactions of most of the compounds including amino acids, peptides, proteins and other primary amines with fluorescamine were essentially complete by the time the reagent front had migrated 10 cm above the origin (Fig. 1). The solvent separates as it moves up the plate. The front contains only hexane and no fluorescamine, which stays with the trailing acetone. Therefore, no reaction occurs during the first 4 cm of migration. It takes 40 min for the hexane front to migrate 10 cm. Less time is required if the plates are dipped in the reagent. Most amines reacted in 5 min whereas amino acids and peptides required 15 min. The protein cytochrome C required 30 min (Fig. 2). Although not evident from a comparison of Figs. 1 and 2, the fluorescence yields obtained by the predipping method (30 min) were equal to those obtained by the predevelopment method (10 cm) (Table I). The yields given in Table I and Fig. 2 were based on the recovery of the compounds reacted in solution, then applied to the plates and similarly treated. 50–70% of the derivatives are recovered from the plate.

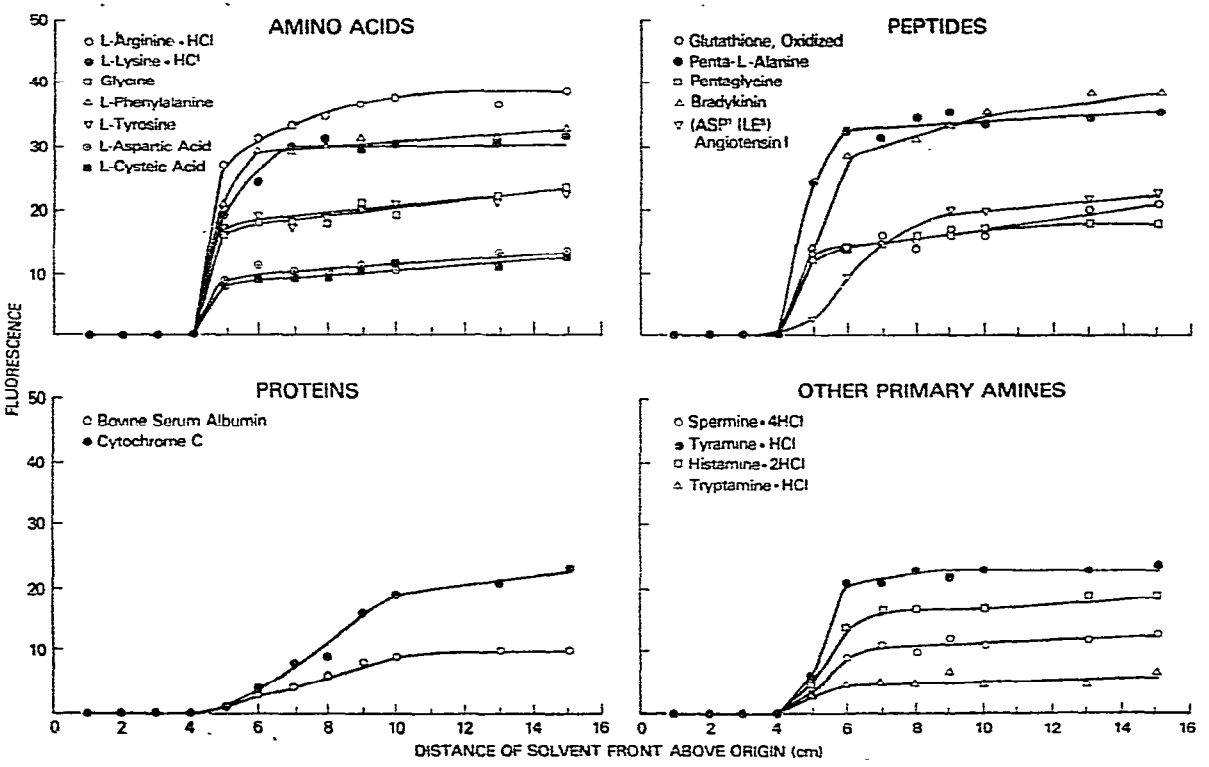

Fig. 1. Derivatization by the predevelopment method. A 1-μl aliquot of the stock solution (1 m*M* or 5 mg/ml) was spotted on the silica gel 60 plate (Merck) and developed for the indicated distance with the derivatization reagent. The fluorescamine derivatives were extracted with 3.5 ml of acetone–0.2 *M* sodium borate pH 9.0 buffer (1:2.5)[7]; the fluorescence was measured in an Aminco-Bowman spectrofluorometer (excitation 390 nm, emission 490 nm).

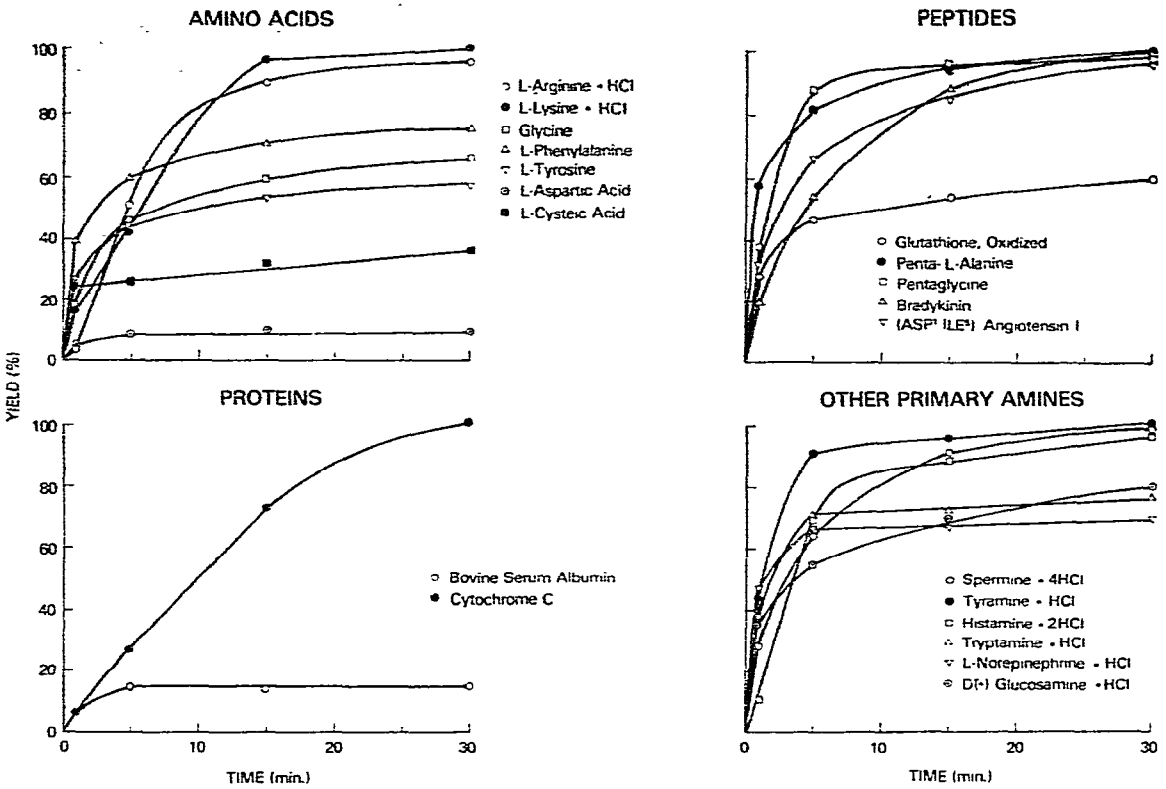

Fig. 2. Derivatization by the predipping method. Yields were calculated as described in Table I and in Experimental.

Sensitivity of the method

Most compounds tested by the predevelopment method were detectable in quantities of less than 10 pmoles (Table II). The exceptions, catecholamines and amino sugars, were detected with equal sensitivity when phosphate buffer was used in place of the borate buffer. Proteins were detected at levels of 0.02–0.03 μg. Equal or slightly higher sensitivity was obtained with the predipping method.

Detection of peptides of biological significance

All the fluorescamine-derivatized peptides stayed at the origin with either the predevelopment or predipping derivatization procedure. Silica gel, alumina or cellulose plates were equally satisfactory for derivatization. Good separations of fluorescamine-derivatized peptides were achieved on the silica gel 60 plates using solvent systems B or C (Table III). R_F values of the derivatives are as reproducible as those of the native compounds. However, cellulose plates tended to give broader and tailing spots with the derivatives. Plastic-backed polyamide layers (Cheng Chin) could not be used because of the presence of interfering fluorescent compounds.

TABLE I

YIELDS OF FLUORESCAMINE DERIVATIVES PREPARED ON SILICA GEL PLATES BY THE PREDEVELOPMENT METHOD

Samples were 1-μl aliquots of a 1-m*M* or 5-mg/ml solution in 0.2 *M* sodium borate buffer (pH 9.0). Test compounds were labeled by developing to a distance of 10 cm above the origin on silica gel 60 plates. Yields were determined by comparing the fluorescence with that obtained with samples reacted with fluorescamine in test tubes, spotted on silica gel plates, dipped in acetone–hexane (1:4) without fluorescamine and similarly treated.

Compound	*Yield (%)*
L-Arginine·HCl	96
L-Cysteic acid	35
L-Aspartic acid	15
L-Lysine	100
L-Phenylalanine	70
Glycine	56
L-Tyrosine	67
Glutathione, oxidized	47
Pentaglycine	78
Penta-L-alanine	84
Bovine serum albumin	8
Cytochrome C	50
Spermine·4 HCl	62
Tyramine·HCl	100
Histamine·2 HCl	75
Tryptamine·HCl	50

TABLE II

R_F VALUES AND LIMITS OF DETECTION OF FLUORESCAMINE DERIVATIVES ON TLC PLATES

Conditions: precoated silica gel 60 TLC plates (Merck) and solvent system A. The predevelopment labeling method was used. Values in parentheses were obtained with compounds dissolved in 0.2 *M* phosphate buffer (pH 8.0). Other compounds were dissolved in 0.2 *M* sodium borate buffer (pH 9.0).

Compound	$R_F \times 100$	*Limit of detection* (10^{-12} *mole**)
L-Arginine·HCl	16	4
L-Cysteic acid	26	9
L-Aspartic acid	26	9
L-Lysine	33	5
L-Phenylalanine	48	6
Glycine	36	6
L-Tyrosine	41	8
Glutathione, oxidized	2	6
Bradykinin triacetate	4	3
Pentaglycine	14	6
Penta-L-alanine	21	6
Bovine serum albumin	0	0.03 μg
Cytochrome C	0	0.02 μg
Spermine·4 HCl	1	7
L-Norepinephrine·HCl	48	80(8)
Tyramine·HCl	67	7
Histamine·2 HCl	36	6
Tryptamine·HCl	70	7
D(+)-Glucosamine·HCl	39	60(7)
D(+)-Galactosamine·HCl	38	70(7)
D(+)-Mannosamine·HCl	38	80(7)

* Unless stated otherwise.

DISCUSSION

Fluorescamine has been used in TLC as both a spray[2–5] and a prelabeling[6] reagent. In the more sensitive (up to ten-fold) prelabeling method, samples are individually reacted with fluorescamine before spotting on TLC plates. Although finding new solvent systems for the separation of the derivatives is not difficult, derivatizing of multiple samples on a microscale is more cumbersome than spraying. The present method was developed to simplify derivatization and increase sensitivity. Fluorescamine-labeling of compounds spotted on TLC plates was achieved with equal effectiveness by developing or dipping the plates in the reagent. Of the numerous amines, amino acids and peptides tested, only β-naphthylamine was displaced from the origin during the labeling procedure. When corrected for the 50–70% recovery from the plates most derivatives were obtained in greater than 50% yield and several in greater than 75% yield by either the predevelopment (Table I) or predipping method (Fig. 2).

TABLE III

SEPARATION OF SOME FLUORESCAMINE-DERIVATIZED PEPTIDES OF BIOLOGICAL INTEREST

Peptides were dissolved in 0.2 *M* sodium borate buffer (pH 9.0) at a concentration of 0.5 m*M*; 1-μl aliquots (5×10^{-10} moles) derivatized by the predevelopment method (10 cm). Precoated silica gel 60 TLC plates (Merck) were used, with the exception of the 4th column (2nd solvent C column) where precoated silica gel plates Q5 (Quantum Industries) were employed.

Peptide	$R_F \times 100$				
	Solvent system				
	B	*C*	*C*	*D*	*E*
L-Carnosine	15	60	68	35	31
L-Homocarnosine sulfate	13	58	68	41	31
L-Anserine nitrate	5	38	69	20	29
Glutathione, oxidized	0	59	71	1	4
Bradykinin	3	16	19	3	25
Lysyl–bradykinin	12	61	76	27	99
Methionyl–lysyl–bradykinin	9	63	79	27	99
Gramicidin J	95	97	98	99	94
Bacitracin	28	89	88	65	99
(Asp[1] Ile[5]) Angiotensin I	4	49	72	29	98
(Asp[1] Ile[5]) Angiotensin II	9	58	76	47	98
Oxytocin	46	89	90	89	99
(Arg[8]) Vasopressin	15	56	61	22	99
Angiotensin II hexapeptide	35	90	87	89	99
Angiotensin II heptapeptide	19	60	78	55	98
Tridecapeptide renin substrate	19	68	86	57	98
Tetradecapeptide renin substrate	15	67	87	60	99
ACTH (porcine)	0	0	1	0	99
Insulin (bovine pancreas)	0	0	1	1	1
Lysine–vasopressin	31	89	89	88	3
Somatostatin tetradecapeptide	34	89	88	89	99
Caerulein	32	88	88	75	99
Secretin 21–27	54	88	87	89	98
Eledoisin-related peptide*	79	94	94	94	98

* L-Lys–L-Phe–L-Ile–Gly–L-Leu–L-Met·NH_2·2 HCl (Sigma).

Notable exceptions were bovine serum albumin, aspartic and cysteic acids (Table I). Presumably some compounds are bound to the silica in such a manner that complete reaction with fluorescamine is not possible. Nonetheless, the sensitivity of the method is at least ten times better than the spraying methods[2–5] and at least as sensitive but much less cumbersome than the other prelabeling method[6].

Replacement of the borate with phosphate buffer greatly increased the sensitivity of detection of catecholamines and amino sugars up to levels comparable to the other amines tested (Table II). Fluorescence quenching may have occurred with these compounds in the borate buffer since borate is known to complex with compounds containing vicinal hydroxyl groups.

Unlike the use of fluorescamine sprays, prelabeling with fluorescamine has not led to problems with amine-containing solvent systems. The high background inherent in the spraying method is also avoided in prelabeling. Stabilization of the fluorescence of the derivatives is achieved by spraying the developed plates with 10% triethanolamine in ethylene glycol monomethyl ether acetate as previously reported[6]. A disadvantage of prelabeling is the restriction to neutral or basic solvent systems for separation of the derivatives because they are unstable in acidic media.

Fluorescamine solvents other than acetone–hexane can be used, depending on the compounds of interest. With amino acids, acetone, dioxane or ethyl acetate (all good solvents for fluorescamine) decrease the reaction time without causing extractive losses. In some studies it may be advantageous to select a fluorescamine solvent which will extract interfering substances during derivatization.

The method has been applied to the TLC analysis of peptides (Table III) and is recommended for its sensitivity and simplicity. This prelabeling procedure also can be used for the specific detection of peptides containing N-terminal tryptophan[8], as well as for the specific detection of tryptophan, tryptamine and certain other indoles by using a 70% perchloric acid spray (manuscript in preparation).

REFERENCES

1 S. Udenfriend, S. Stein, P. Böhlen, W. Dairman, W. Leimgruber and M. Weigele, *Science*, 178 (1972) 871.
2 A. M. Felix and M. H. Jimenez, *J. Chromatogr.*, 89 (1974) 361.
3 M. Furlan and E. A. Beck, *J. Chromatogr.*, 101 (1974) 244.
4 J. Sherma and J. C. Touchstone, *Anal. Lett.*, 7 (1974) 279.
5 B. Klein, J. E. Sheehan and E. Grunberg, *Clin. Chem.*, 20 (1974) 272.
6 K. Imai, P. Böhlen, S. Stein and S. Udenfriend, *Arch. Biochem. Biophys.*, 161 (1974) 161.
7 H. Nakamura and J. J. Pisano, *Arch. Biochem. Biophys.*, 172 (1976) 102.
8 H. Nakamura and J. J. Pisano, *Arch. Biochem. Biophys.*, 172 (1976) 98.