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# AN IMPROVED AND EASY TECHNIQUE FOR POLYAMINE DETERMINATION IN BIOLOGICAL SAMPLES

## APPLICATION TO CELL-FREE SYSTEM FROM HYPERTROPHIED RAT HEART

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SUMMARY

An accurate, improved cation-exchange chromatographic method using *o*-phthalaldehyde and ultraviolet detection at 280 nm for the determination of free polyamines (putrescine, spermidine, spermine) has been developed. Different samples, such as the 105,000 *g* supernatant of reticulocyte or heart muscle, and KCl ribosomal wash containing initiation factors, can be analysed. The minor modification of reagents results in a good precision and sensitivity, which is demonstrated by a relative standard deviation of 5—9% and recoveries of 98%. This technique is of particular interest because it allows polyamine determination in biological samples with high concentrations of salt.

## INTRODUCTION

In many situations such as heart hypertrophy, cell growth, or regenerating rat liver, the polyamine pool increases within a few hours of either chemical or pharmacological treatment [1, 2].

Polyamines are involved in many steps of the replication and transcription processes. Because of their polyanionic nature they interact with DNA and RNA and increase protein synthesis in cell-free systems [3—6]. The stimulatory effects occurring under such conditions could be due to a rapid accumulation of newly synthesized free polyamines [7].

Therefore the relationship between these molecules and the events involved in the regulation of cell growth or differentiation requires an improvement of the sensitivity of the analytical and quantitative techniques for polyamine determination in biological samples.

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High-speed amino-acid autoanalysers coupled with continuous fluorescence monitoring allow a good quantitative separation [8—11]. Other methods such as gas chromatography, mass spectrometry or radioimmunoassay [12] are fast and sensitive. However, all these techniques require sophisticated equipment which might not be available in all laboratories for routine analysis. Moreover, common quantitation techniques involving butanol extraction before high-voltage paper electrophoresis or thin-layer chromatography, followed by specific staining, are very dependent on ionic strength and might result in an underestimation of the amount of polyamine [13].

This paper describes a simple specific method which requires neither expensive instrumentation nor many extraction steps. We have developed an easy, efficient procedure which can be applied to biological samples of high ionic strength, utilizing a cation-exchange chromatographic method for separation, and *o*-phthalaldehyde (OPA) for detection (because of its good aqueous solubility and high sensitivity), followed by detection of the reaction by their absorbance at 280 nm.

The sensitivity of this method was sufficient to enable us to establish a relationship between induction of cardiac hypertrophy induced by L-triiodothyronine ($T_3$) treatment and the amount of free polyamines in different cell compartments.

## EXPERIMENTAL

### *Reagents*

Putrescine, sarcosine, spermidine and spermine from Sigma (St. Louis, Mo., U.S.A.) were dissolved in water to make 10 m*M* stock solutions which were kept at 4°. More dilute solutions were not stable. All reagents used for the buffers were from Merck (Darmstadt, G.F.R.), except *o*-phthalaldehyde (OPA), which was obtained from Sigma or from Fluka (Buchs, Switzerland), and fluorescamine which was a gift from Roche (Basel, Switzerland). Buffers for the fluorescamine solution and reaction were prepared according to the description of Felix et al. [14]. The method and buffer when OPA was used, were as described in refs. 15 and 16 with some modifications.

For the assays with different buffers and pH conditions the reagent was prepared with 1 *M* boric acid, adjusted to pH 10.3 with 2 *M* potassium hydroxide, then β-mercaptoethanol was added to 1:1000, and Brij 30% to 1.5:1000. The OPA, dissolved in a few millilitres of methanol, was diluted in the buffer to a final concentration of 40 mg/ml. Under these conditions the pH is kept at 9.8—10.

The reaction mixture has a final volume of 2 ml; 1 ml of the reagent solution is added to the samples of polyamines. Phosphate or pyridine buffer is, in some cases, added to obtain the desired pH. The volume of the sample never exceeds 0.5 ml.

For chromatography and assays with KCl, OPA is at a concentration of 125 mg/ml in 0.5 *M* boric acid dissolved in 2 *M* potassium hydroxide to give pH 10.2. Brij 30% is then added at 1.5:1000 and β-mercaptoethanol at 1:1000, then 0.16 *M* pyridine—acetic acid buffer from a 2 *M* (pH 5.5) stock solution is added to obtain a final pH of 8.65—8.7. The stability under nitrogen is very

good for many days. After 45 min reaction, the loss of absorbancy was 2% lower than after 25 min.

*Tissue extracts, preparation of polysomes and initiation factors*

Polysome and reticulocyte initiation factors were prepared as described previously [17]. The ribosomes and soluble fractions from normal and treated rat hearts (daily intraperitoneal injection of $T_3$ at the dose of 15 $\mu$g per 100 g) were prepared as previously described [18], except that phosphate buffer (pH 7.6) was used instead of Hepes buffer.

The initiation factors were prepared according to the method of Heywood and Rich [19]; however, the ribosomes were washed for 1 h at 4°.

*Polyamine extraction*

The supernatant and the crude initiation factors were dialysed for 24 h at 4° against 20 volumes of distilled water. The dialysate was lyophilized and redissolved in water. KCl was precipitated with 1 *M* perchloric acid while the pH was kept above 4.0, and removed by centrifugation at 2500 *g* for 10 min. An aliquot of the supernatant was applied to a Dowex column.

*Chromatography*

A 0.5 × 9 cm column was packed with Dowex 50W-X4, 200—400 mesh, from Bio-Rad Labs. (Richmond, Calif., U.S.A.). The first step of the analysis of the polyamines on this cation-exchange column is as described by Katz and Comb [20] for the separation of nucleosides. The experimental procedure is given in Fig. 1A and B. After regeneration of the column, the sample is applied onto the column in 0.05 *M* HCl. The column is washed for 0.5 h with water then with 20 m*M* phosphate (pH 8.0) for 0.5 h, and finally equilibrated with 20 m*M* phosphate—0.5 *M* KCl buffer for 1 h. The polyamines are eluted with 30 ml of a linear KCl gradient from 0.5 to 3.0 *M* in 20 m*M* phosphate buffer. Putrescine is eluted at 1.3 *M* KCl, spermidine at 2.25 *M* KCl and spermine at 2.75 *M* KCl (Fig. 1B).

Automatic monitoring was obtained, as shown in Fig. 1A, using an Isco spectrophotometer (absorbance monitor U $A_4$) with a standard flow-cell of 76 $\mu$l illuminated volume and an Isco 950 digital integrator.

## RESULTS AND DISCUSSION

*Recovery of polyamines and accuracy*

Known amounts of polyamines were loaded on the Dowex column and eluted. The areas under the peaks were determined with the 950 Isco integrator. Areas and concentrations are related in Fig. 2. The standard plots in Fig. 2 were used afterwards in all the biological experiments. The relative standard deviation is less than 9% for putrescine, 7% for spermidine and 5% for spermine. Recovery was between 90 and 98% and was determined by adding known amounts of polyamines to the biological samples. Reproducibility is good when the ionic concentration does not exceed 1.0 *M*, whatever the biological fluid. Other amines such as lysine, arginine or sarcosine do not interfere.

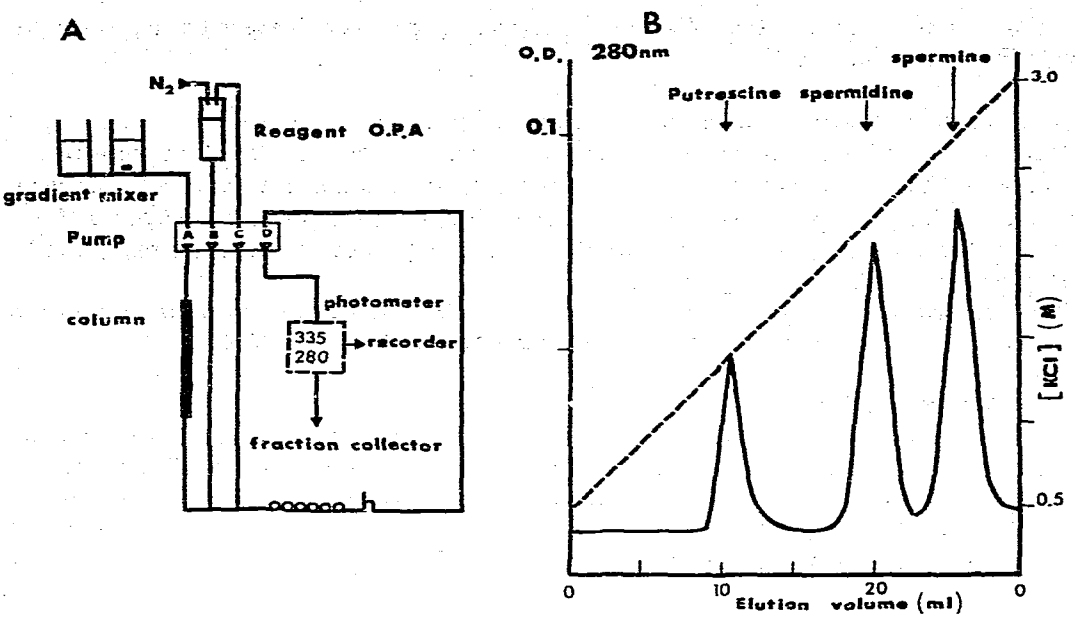


Fig. 1. Calibration experiments: chromatography on Dowex 50W-X4 with automatic monitoring at 280 or 335 nm. (A) Experimental procedure. Flow-rates: gradient mixer (A), 9.54 ml/h; reagent (B), 19.2 ml/h; nitrogen (C), 9.54 ml/h; final effluent (D), 25.2 ml/h. (B) Elution curve obtained by cation-exchange liquid chromatography of a calibration mixture of 62.5 nmoles of each polyamine. Elution gradient was 0.5—3.0 *M* KCl in 20 m*M* phosphate.

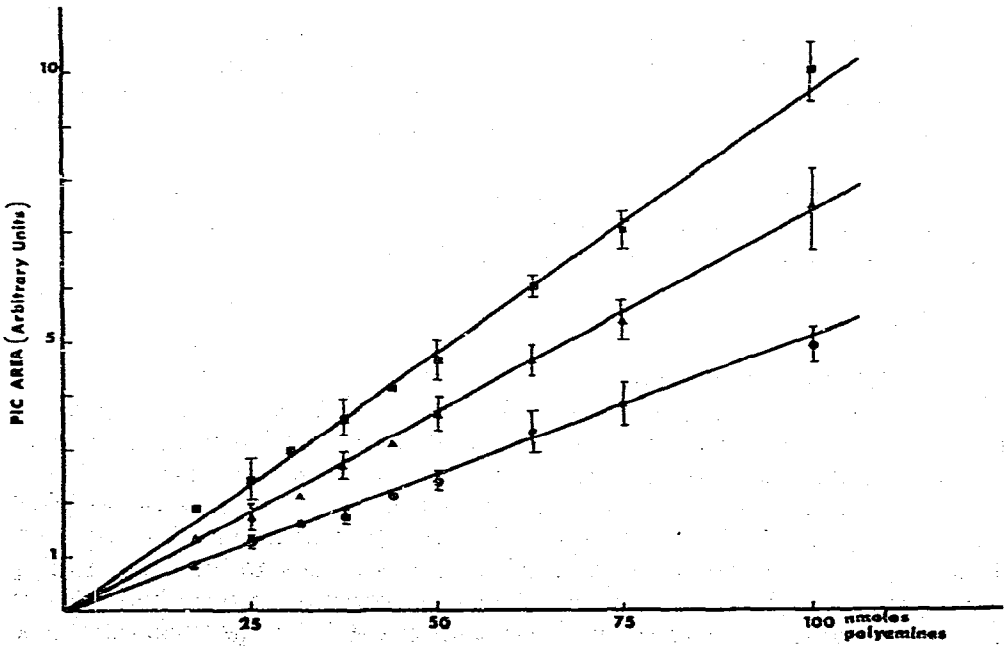


Fig. 2. Standard curves for determination of polyamines: quantitation of putrescine ( ● ); spermidine ( ▲ ); and spermine ( ■ ). Peak areas (expressed in arbitrary units) were plotted against known amounts of polyamines (in nmoles).

*Choice of reagents and wavelength*

According to Benson and Hare [21], OPA in the presence of 2-mercaptoethanol reacts with primary amines to form highly fluorescent products, the nature of which has not yet been determined. In contrast, neither OPA nor fluorescamine forms fluorophors with secondary amines.

We have examined the reaction of fluorescamine and OPA with three polyamines of biological interest; putrescine, spermidine and spermine. The two reagents have been compared for sensitivity and reaction rates when used in tenfold excess: OPA is seven and five times more sensitive than fluorescamine with spermidine and spermine, respectively (Fig. 3). Similar results have been published by other authors [9, 21, 22].

Fig. 4 shows that spermidine and spermine have two significant absorbance levels, at 335 nm, used for fluorescence detection, and at 280 nm. The molar

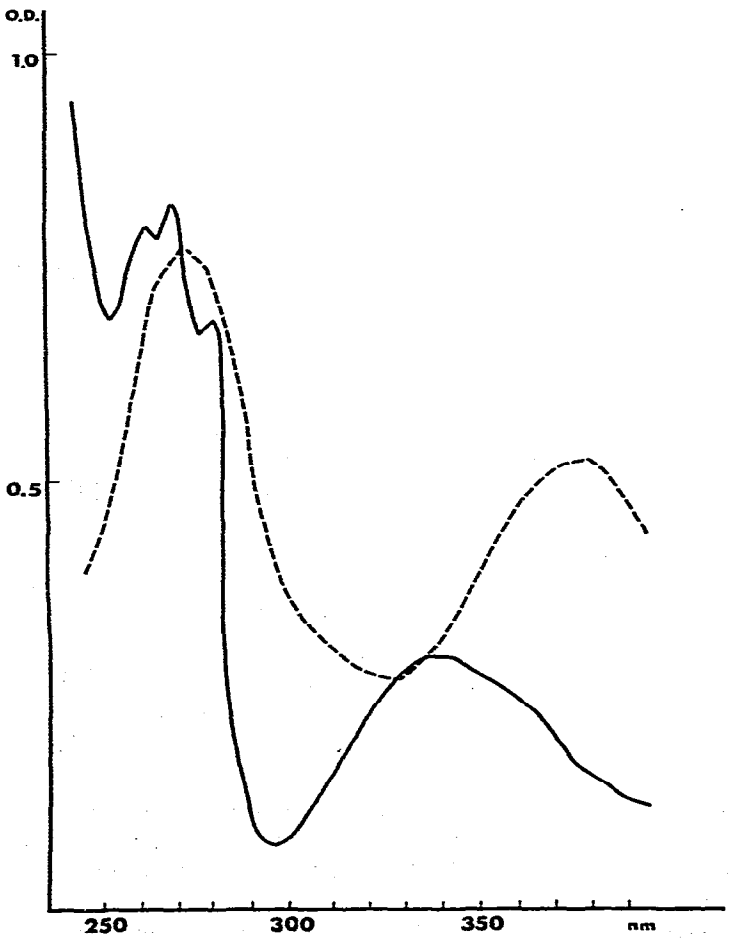


Fig. 3. Ultraviolet and visible spectra of reaction products of spermine $5.0 \times 10^{-4}$ *M* with fluorescamine ( - - - - ) and spermine $1 \times 10^{-4}$ *M* with *o*-phthalaldehyde (———). Fluorescamine and OPA are in tenfold excess. Spectra are recorded against blanks containing reagents alone. Equilibrium is reached immediately with fluorescamine and after 15 min with OPA.

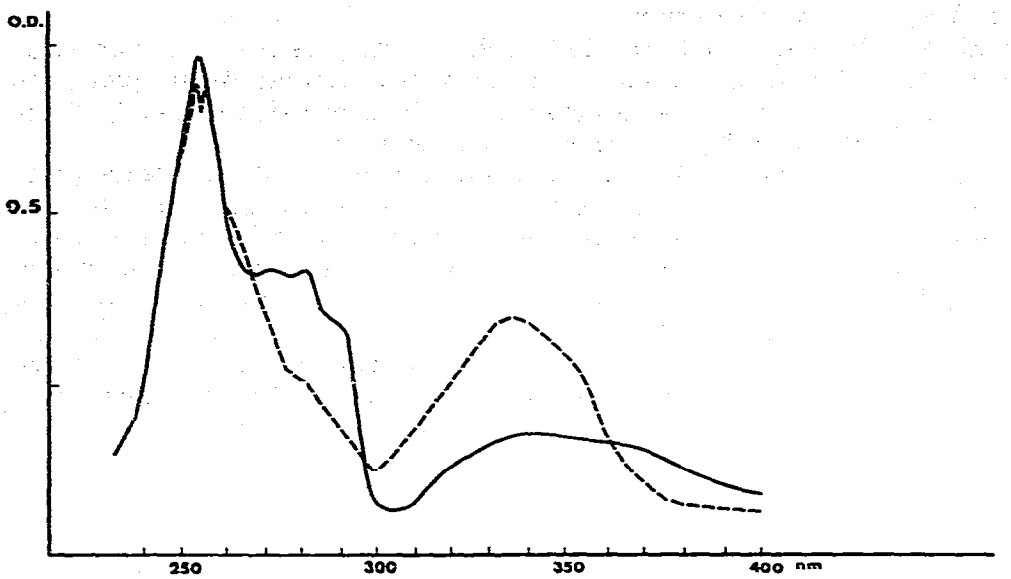


Fig. 4. Ultraviolet and visible spectra of the reaction products of o-phthalaldehyde with spermine $1 \times 10^{-4}$ *M* (- - - - -) and spermidine $1 \times 10^{-4}$ *M* (———).

extinction coefficient after 20-min reaction of OPA and the three polyamines (Table I), indicates that for spermine in particular, the sensitivity is higher at 280 nm than at 335 nm.

*Kinetics of the reaction in phosphate buffer*

In 50 m*M* phosphate (pH 8.0, which gave a pH of 9.8 in the final reaction mixture), with each of the three polyamines examined we observed a hypochromicity at 280 nm and at 335 nm, both reaching a plateau after 20—25 min (Figs. 5—7).

*Kinetics of the reaction in pyridine buffer*

As polyamines were isolated on a CM-cellulose column, eluted with a 0—0.5 *M* pyridine—acetic acid gradient, it was necessary to determine the influence of this buffer on the OPA reaction [9]. There is still hypochromicity at 335 nm with spermine and spermidine (Figs. 5 and 6), but a hyperchromic effect appears with putrescine at this wavelength (Fig. 7). A plateau is reached faster than in phosphate buffer.

All these results, summarized in Table I, show that the best sensitivity for the determination of spermine in pyridine buffer is obtained at 280 nm, whereas for spermidine and putrescine there is little difference compared with determination in phosphate buffer.

The major advantage is the better sensitivity in spermine determination, which is the more interesting problem from a biological point of view.

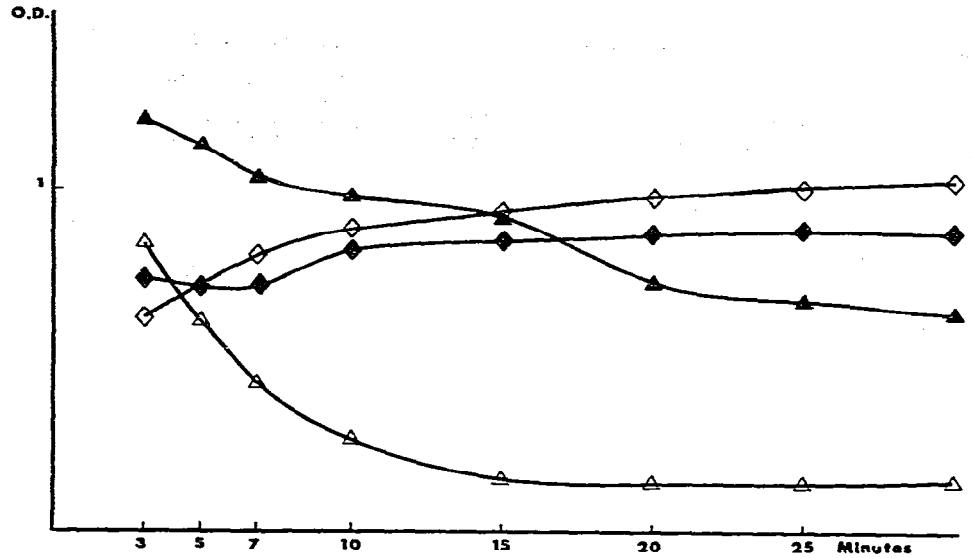


Fig. 5. Kinetics of the reaction of spermidine $1 \times 10^{-4}$ *M* with tenfold excess of OPA. Phosphate buffer (50 m*M*, pH 8.0; final pH 9.8) at 335 nm (▲) and 280 nm (◆). Pyridine—acetic acid buffer (250 m*M*, pH 5.0; final pH 8.6) at 335 nm (△) and at 280 nm (◇).

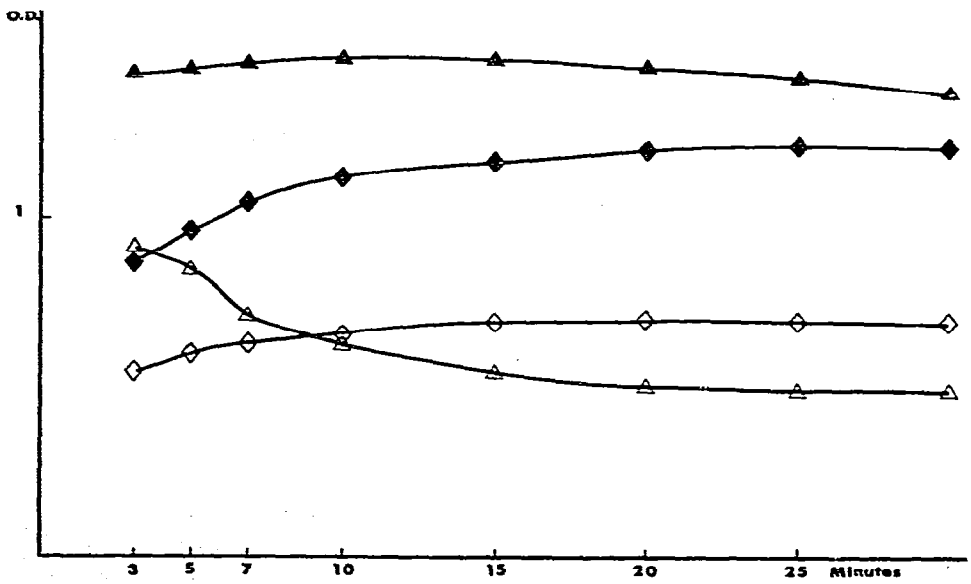


Fig. 6. Kinetics of the reaction of spermidine $1 \times 10^{-4}$ *M* with OPA. Phosphate buffer (50 m*M*, pH 8.0; final pH 9.8) at 335 nm (▲) and at 280 nm (◆). Pyridine—acetic acid buffer (250 m*M*, pH 5.0; final pH 8.6) at 335 nm (△) and at 280 nm (◇).

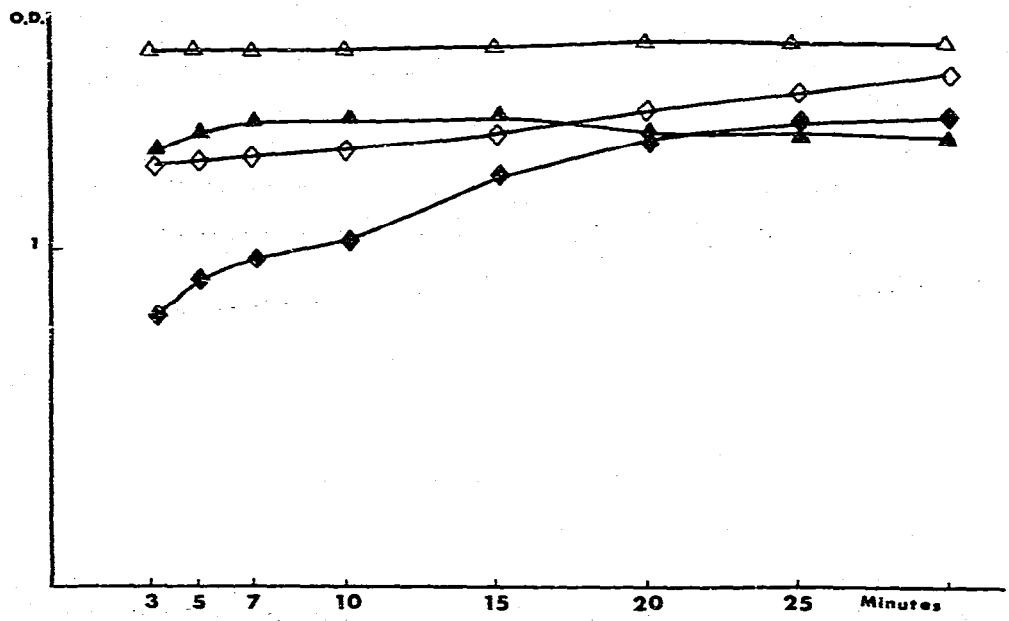


Fig. 7. Kinetics of the reaction of putrescine $1 \times 10^{-4}$ *M* with OPA. Phosphate buffer (50 m*M*, pH 8.0; final pH 9.8) at 335 nm (▲) and at 280 nm (◆). Pyridine—acetic acid buffer (250 m*M*, pH 5.0; final pH 8.6) at 335 nm (△) and at 280 nm (◇).

TABLE I

COMPARISON OF OPA REACTION PRODUCTS WITH POLYAMINES IN TWO BUFFERS AND AT TWO WAVELENGTHS

Reaction time, 20 min. Buffers: 50 m*M* phosphate (final pH 9.8); pyridine—acetic acid, 250 m*M* (final pH 8.6).

| | Molar absorbance index ($M^{-1} \cdot cm^{-1} \cdot 10^{-5}$) | | | |
|---|---|---|---|---|
| | Phosphate buffer | | Pyridine buffer | |
| | 335 nm | 280 nm | 335 nm | 280 nm |
| Putrescine | 63.5 | 70 | 78.5 | 60 |
| Spermidine | 59 | 48 | 24.5 | 31 |
| Spermine | 35 | 37.5 | 14.5 | 50.5 |

*Influence of ionic strength, pH, and nature of the ions*

The ionic strength conditions are those of the chromatographic conditions on Dowex 50W-X4, and elution by a linear 0.5—3.0 *M* KCl gradient (Fig. 1B). Actually, this chromatographic procedure was chosen because it was not possible to use CM-cellulose without butanol extraction when analysing biological samples.

*20 mM phosphate buffer with KCl.* It can be seen from Fig. 8 and Fig. 9A that strong hypochromicity at 280 nm is accompanied by hyperchromicity at 335 nm for spermine and spermidine.

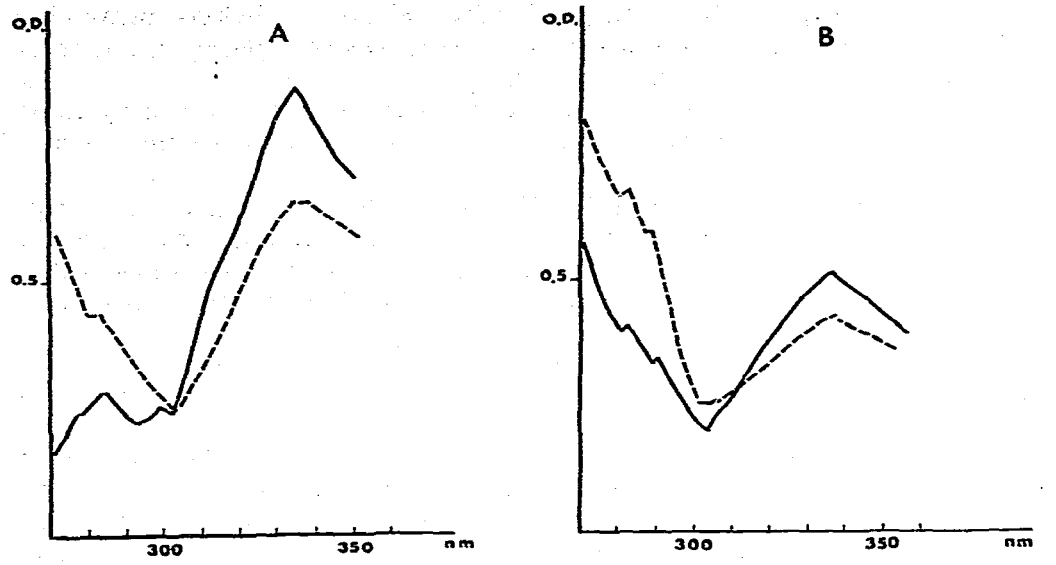


Fig. 8. Ultraviolet and visible spectra in 20 m*M* phosphate buffer, of reaction products of OPA with: (A) spermidine $1 \times 10^{-4}$ *M* with 2.0 *M* KCl (———), without KCl ( - - - - -); and (B) spermine $1 \times 10^{-4}$ *M* with 3.0 *M* KCl (———), without KCl ( - - - - -).

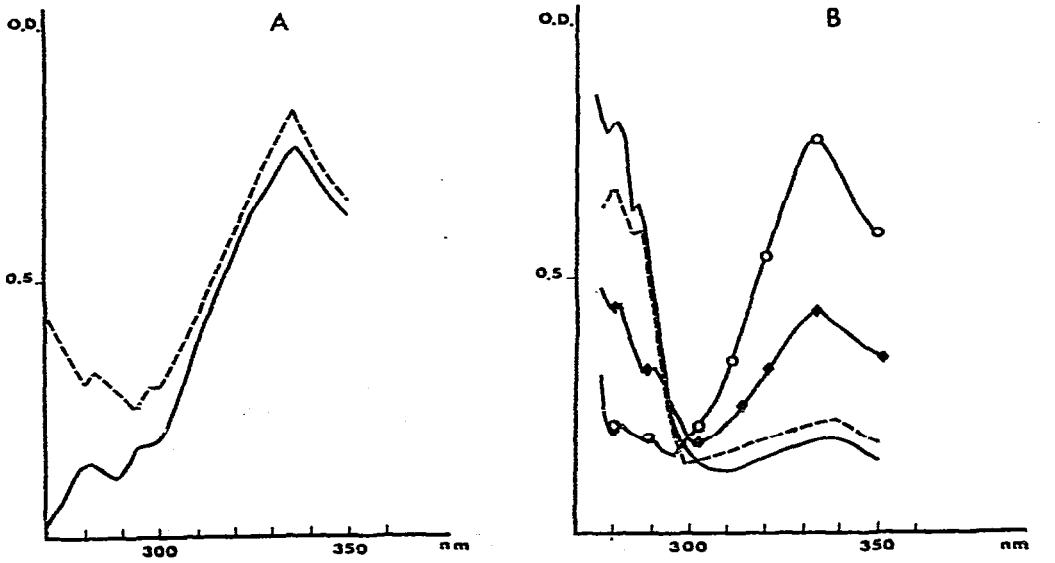


Fig. 9. Ultraviolet and visible spectra of reaction products of OPA with: (A) putrescine, $1 \times 10^{-4}$ *M* in 20 m*M* phosphate buffer, with 1.5 *M* KCl ( ——— ), without KCl ( - - - - - ); (B) pyridine—acetic acid 160 m*M* (final pH 8.5) (polyamine concentration $1 \times 10^{-4}$ *M*). (○), Putrescine with or without KCl, 1.5 *M*; (▪), spermidine with or without KCl, 2.0 *M*; ( - - - ), spermine with 3.0 *M* KCl; ( ———), spermine without KCl.

When varying the pH with acetic acid in the above conditions, putrescine has a higher absorbancy at a slightly alkaline pH, whereas spermine and spermidine absorb more at a higher pH (Fig. 10A).

As the data presented above showed better results with the use of pyridine buffer, we examined the effect of the addition of pyridine to the 20 m*M* phosphate—KCl buffer.

*Action of 20 mM phosphate—160 mM pyridine buffer.* At pH 10.0 better reactivity was obtained at 160 m*M* pyridine with a good stability. When the pH is varied in the presence of 160 m*M* pyridine, the maximum absorbance at 280 nm is obtained at pH 8.5 for spermine and spermidine, whereas no change occurs with putrescine (Fig. 10B).

Under these conditions (pH 8.5, 20 m*M* phosphate, 160 m*M* pyridine—acetic acid) large concentrations of KCl have almost no influence on the spectra, as shown in Fig. 9B.

The decrease in molar extinction coefficient at 335 nm for the reaction of spermine with OPA in phosphate buffer alone could be due to an influence of secondary amino groups on the reaction with primary amino groups. The new reaction products will be more stable in the presence of pyridine buffer at 280 nm.

Therefore, while the three polyamines are eluted with different KCl concentrations, the conditions determined above allowed quantitative analysis after a simple and reproducible separation on a column of Dowex 50W-X4 ion-exchange resin.

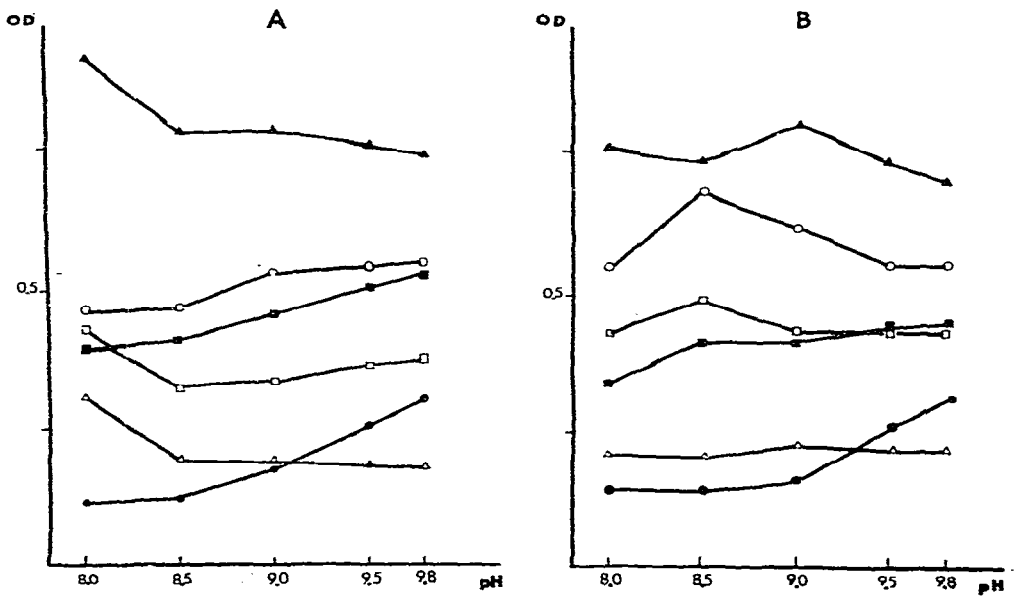


Fig. 10. Effect of pH on absorbance of the reaction products of OPA (at 335 nm closed symbols, at 280 nm open symbols) with putrescine (▲), spermidine (■), and spermine (●), (A) in the presence of 20 m*M* phosphate buffer, (the pH was adjusted with acetic acid) and (B) in the presence of 160 m*M* pyridine (the pH was adjusted with acetic acid or potassium hydroxide).

*Application to the determination of free polyamine levels in three cases*

The procedure described is particularly useful for biological samples containing a high salt concentration as, for example, a 0.5 *M* KCl ribosomal wash, in which the polyamine concentrations have been determined successfully by this rapid ion-exchange technique.

*Reticulocyte polyamines.* No free putrescine was detected in the different cell fractions with the experimental procedure. This confirms the results of other authors [23] (Table II). The extraction of free polyamines by dialysis becomes quantitative when the ribosomal wash protein concentration is lower than 20 mg/ml. If the concentration is higher, the spermidine—spermine ratio in the dialysate is modified.

The amount of polyamines found in the initiation factors corresponds to 35 nmoles per mg of ribosomes. The results have been confirmed with biological tests in a cell-free system: the amount of polyamines needed for maximal activity in a cell-free protein synthesis assay [6] corresponds exactly to the amount found after dialysis of the crude initiation factors (unpublished results).

This confirms that the simple technique described here is well adapted to the determination, with a good reproducibility, of free cytoplasmic polyamines that play a fundamental role in the control of protein synthesis.

*Rat heart polyamines.* No free polyamines were detectable in the crude initiation factors, perhaps because the amount of myosin present in our preparations retains some polyamines in a non-specific way. However, accumulation of newly synthesized polyamines can be detected by this experimental procedure. The level of each polyamine is comparable to other published data [24] (Table II). Spermidine—spermine ratios in the supernatants of reticulocytes and muscle show great differences.

*Polyamine levels during $T_3$-induced rat heart hypertrophy in high-speed supernatants.* Just after the first injection, the level of free polyamines synthesized increases, then it decreases over a period of six days (Fig. 11); but the spermidine—spermine ratio shows that the synthesis rates are different. During treatment there is no accumulation of putrescine, and particularly at 6 and 12 h.

TABLE II

SPERMIDINE AND SPERMINE CONCENTRATIONS IN DIFFERENT CELLULAR COMPARTMENTS

| | Spermidine | Spermine |
|---|---|---|
| Reticulocyte | | |
| Initiation factors (nmoles/mg protein) | 35.2 | 3.85 |
| Supernatant (nmoles/mg protein) | 1.5 | 0.12 |
| Cardiac muscle supernatant | | |
| (nmoles/g) | 91 | 50.5 |
| (nmoles/mg protein) | 3.8 | 1.9 |

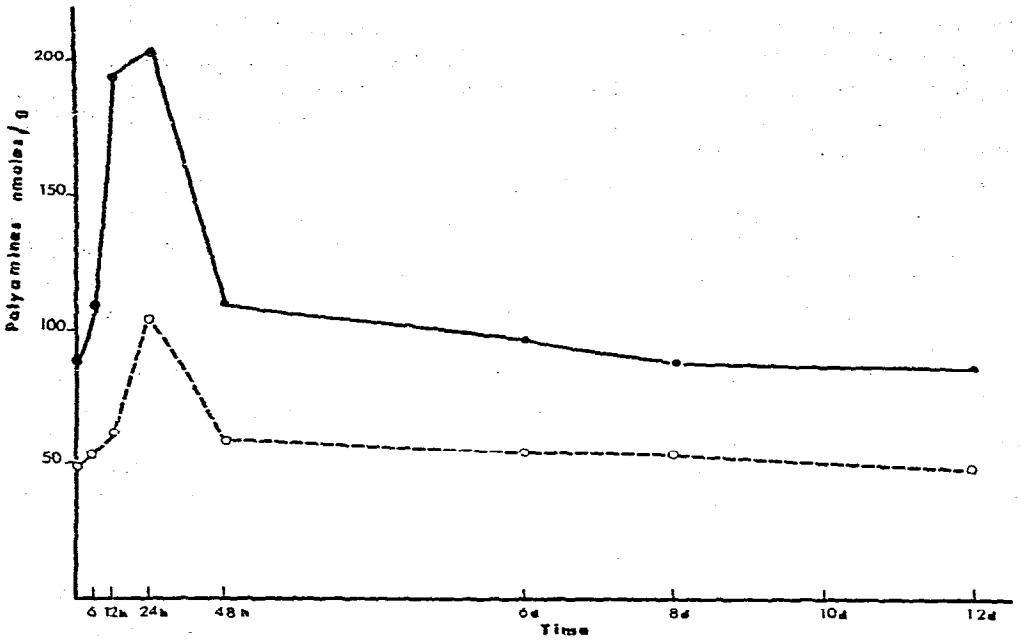


Fig. 11. Polyamine concentration in rat heart post-ribosomal supernatant during triiodothyronine treatment. (●—● ), spermidine, (o---o ), spermine.

It has been shown that after any cellular modifications following administration of drugs the spermidine level increases more than the spermine level, and maximal activity of the amine biosynthesis pathway is quickly reached [25, 26].

For example, $T_3$ treatment produces a rapid increase of heart ornithine decarboxylase activity and this first enzyme in polyamine synthesis has a short half-life [27].

## CONCLUSION

The procedure developed to determine polyamines is an easy, efficient routine cation-exchange chromatographic method, highly sensitive and reproducible, using a common spectrophotometer (absorbance at 280 nm) and a common reagent, *o*-phthalaldehyde.

This improved technique is particularly useful in the measurement of free polyamines from dialysed salt extracts, as for example the 0.5 *M* KCl ribosomal wash and high-speed supernatant. It does not involve several tedious isolation steps and enzymes can be studied with the same preparation.

The concentration of free polyamines in reticulocytes, cells and heart muscle have been determined successfully by this rapid chromatographic procedure and demonstrates the utility of the method for any biological samples, even in the presence of high ionic-strength buffer.

ACKNOWLEDGEMENTS

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REFERENCES

1 H. Tabor and C.W. Tabor, Adv. Enzymol., 36 (1972) 203.
2 P. Antony, K. Gibson and P. Harris, J. Mol. Cell. Cardiol., 8 (1976) 589.
3 L. Stevens, Biochem. J., 103 (1967) 811.
4 J.F. Atkins, J.B. Lewis, C. Anderson and R.F. Gesteland, J. Biol. Chem., 250 (1975) 5688.
5 M.N. Thang, L. Dondon and E. Mohier, FEBS Lett., 61 (1976) 85.
6 M. Raymondjean, D. Bogdanovsky, L. Bachner, B. Kneip and G. Schapira, FEBS Lett., 76 (1977) 311.
7 A. Kallio, H. Poso, S. Guma and J. Janne, Biochem. J., 166 (1977) 89.
8 N. Seiler, J. Chromatogr., 143 (1977) 221.
9 K. Samejima, M. Kawase, B. Sacamoto, M. Okada and Y. Endo, Anal. Biochem., 76 (1977) 392.
10 C.W. Gehrke, K.C. Kuo, R.L. Ellis and T.P. Waalkes, J. Chromatogr., 143 (1977) 345.
11 H. Adler, M. Margoshes, L.R. Snyder and C. Spitzer, J. Chromatogr., 143 (1977) 125.
12 F. Bartos, D. Bartos, D.P. Grettie, R. Campbell, L.J. Marion, R.G. Smith and G. Daves, Biochem. Biophys. Res. Commun., 75 (1977) 915.
13 O. Heby and G. Andersson, J. Chromatogr., 145 (1978) 73.
14 A.M. Felix, U. Toome, S. Debernardo and M. Weigele, Arch. Biochem. Biophys., 168 (1975) 601.
15 M. Roth, Anal. Chem., 43 (1971) 880.
16 L.E. Lund, J. Thomsen and K. Brunfeldt, J. Chromatogr., 130 (1977) 51.
17 D. Bogdanovsky, W. Hermann and G. Schapira, Biochem. Biophys. Res. Commun., 54 (1973) 25.
18 L. Bachner, M. Raymondjean, D. Bogdanovsky, B. Kneip and G. Schapira, Biochimie, 59 (1977) 863.
19 S.M. Heywood and A. Rich, Proc. Nat. Acad. Sci. U.S., 59 (1968) 590.
20 S. Katz and D.G. Comb, J. Biol. Chem., 238 (1963) 3065.
21 J.R. Benson and P.E. Hare, Proc. Nat. Acad. Sci. U.S., 72 (1975) 619.
22 L.J. Marton and P.L.Y. Lee, Clin. Chem., 21 (1975) 1721.
23 D. Konecki, G. Kramer, P. Pinphanichakarn and B. Hardesty, Arch. Biochem. Biophys., 169 (1975) 192.
24 M.J. Feldman and D.H. Russell, Amer. J. Pathol., 222 (1972) 1199.
25 A. Kallio, H. Poso, S. Guha and S. Janne, Biochemistry, 166 (1977) 89.
26 T. Eloranta and A. Raina, Biochem. J., 168 (1977) 179.
27 P. Antony, K. Gibson and P. Harris, J. Mol. Cell. Biol., 8 (1976) 589.