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EVIDENCE FOR THE EXISTENCE OF cAMP IN LILY PLANT FLOWER TISSUES

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Key Word Index—*Lilium*; Liliaceae; pollination; long-distance signalling; adenylyl cyclase; radioimmunoassay; cAMP.

Abstract—A specific radioimmunoassay (RIA) has been developed for the quantitative measurement of the cAMP content of floral organs of *Lilium* cv. Connecticut. Significant quantities of cAMP were detected in and around the stigma (highest specific activity), style, ovaries and anthers, but not in the tepals or filaments. The specificity of the method was established by treating the extract of the upper part of the style (including the stigma) with bovine spleen phosphodiesterase. This led to a 50% loss of the cAMP content of the extract. Variations in the cAMP content of this organ were also observed 30 min after flower self-pollination and 90 min after tepal wounding. The presence of cAMP in the lily flower was confirmed by measuring the adenylyl cyclase activity in the membrane fraction of an extract of the upper part of a style (including the stigma). This activity was stimulated by forskolin and aluminium fluoride salts. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Even though adenosine 3′,5′-cyclic monophosphate (cAMP) has been well defined as a second messenger in animal cells [1] and a primary messenger in bacteria [2], its role in plants is still controversial (reviewed in Assmann [3]). Enzymic activities similar to the ones involved in cAMP metabolism in animal cells have been observed in several plant species. These include adenylyl cyclase [4–6], phosphodiesterase [7, 8] and cAMP-dependent protein kinase activity [9, 10]. cAMP content has also been quantified in over 30 plant species (reviewed in Newton and Brown [11]), and the extracellular addition of cAMP on plant tissues has even been shown to affect several cellular processes [12–18]. However, in the latter studies cAMP detection has often been incorrect due to several artefacts leading to large overestimates. The possible origin of cAMP from bacterial contaminants has even led to a debate about whether it is really produced by plant cells [19] and, rendering the picture surrounding cAMP even more unclear, virtually no quantitative study has shown any significant change in cAMP content during the signal transduction events under scrutiny, thus weakening the claim that cAMP plays a role as a messenger in plants. Nevertheless, cAMP has now been isolated from several plant tissues and further identified by mass spectroscopy [12, 20].

The control by cAMP of self-incompatible pollen tube growth in the lily flower is one of the recently described cellular processes worthy of further scrutiny [3]. Of all the physiological processes in which cAMP has been implicated, it is indeed one of the rare systems which can be activated by the exogenous addition of low (and probably physiological) doses of cAMP. It has actually been shown that the extracellular addition to cut styles of as little as 10 nM cAMP was able to rescue the growth of self-incompatible pollen tubes in this species [17]. Drugs known to elevate cAMP levels in animal cells such as forskolin or 1-isobutyl-3-methylxanthine were also able to initiate the same process. However, no study has yet supported the existence of cAMP molecules in plant flowers or of cAMP-generating systems in the tissues involved in the control of self-incompatible pollen tube growth.

Using a combination of estimating the cAMP content of whole tissues radioimmunoassay (RIA) and measuring the adenylyl cyclase activity in isolated

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membranes, we have obtained evidence to show that cAMP is present in the upper style, including the stigma, of the lily flower. Changes in the cAMP were also observed in this tissue after flower self-pollination and after tepal wounding.

RESULTS AND DISCUSSION

The cAMP content of six floral tissues of *Lilium* cv. Connecticut was estimated by RIA and expressed in terms of femtomoles of cAMP per milligram fresh weight and femtomoles of cAMP per milligram of protein (Table 1). Significant quantities of cAMP were detected in all tissues, but in the filaments and tepals the concentration was too close to the detection method limits (about 1 fmol/mg fresh weight of tissue) to be significant. The yield of cAMP was estimated to be 74.5±2.4% ($n = 3$) using radio-iodinated cAMP as standard. The cAMP content of the anthers cannot easily be compared with the values calculated for other tissues because of the high dehydration factor of this organ and its high storage protein content. The values are nevertheless significant and seem to be associated with the pollen grains, since no significant quantity of cAMP could be detected in the anther envelopes (data not shown). In all cases it was necessary to measure the cAMP and protein content in the same sample in order to obtain reproducible results. For later experiments, the results will be expressed in terms of femtomole of cAMP per milligram of protein because of the important variations in the water content of the tested tissues between individual sets of cut flowers and on opening of the flowers.

Using a similar RIA technique it was found that, after eliminating artefactual sources of cAMP and possible interfering substances, the cAMP levels in the seeds, calli and embryos of several plant species were below the detection limit of 0.5 pmol/g.fresh weight of tissue [19]. However, using the same technique more recent studies on *Torenia* stem fragments [21], *Phaseolus vulgaris* cell suspension cultures [22] and *Lemna* axenic cultures [23] revealed significant cAMP concentrations which are in the same concentration range as the ones seen in the plant flower sexual organs. The present results on the cAMP content of lily flower tissue agree with all the results of these studies and support the idea that cAMP production may be limited to some plant tissues, since even in the same flower large variations in cAMP content are observed. cAMP accumulation may also depend on the physiological state of the tissue, as higher cAMP levels were seen in lily flower stigmas during anthesis, while being virtually undetectable in green flower buds (data not shown). Nevertheless, the possible existence of artificial substances in our extracts cannot be ruled out and deserves close attention.

Table 1. cAMP content of various tissues of the lily flower*

Tissue	cAMP content fmol/mg fresh weight	cAMP content pmol/mg protein
Anthers	8.9±0.4	0.61±0.09
Filaments	0.9±0.1	0.26±0.09
Ovaries	5.9±0.3	0.45±0.07
Style (upper part)†	15.1±5.9	1.32±0.61
Style (lower part)	4.3±0.7	0.64±0.14
Tepals	0.8±0.4	0.17±0.09

*Flowers were dissected and each tissue weighed and ground in perchloric acid. Protein content was then estimated using the precipitate and the cAMP concentration using the supernatant, as described in the Experimental. Each value represents the mean±S.D. of two estimates obtained using two different flowers taken from different flower batches. Each estimate also corresponds to two measurements made on the same flower extract.

†Containing stigma.

In order to test the specificity of our detection method, we first measured the cAMP content of a two-fold dilution of the tissue extract. As shown in Table 2 on a top style section extract, a two-fold dilution of this extract led to a measured two-fold lower cAMP content. A similar result was obtained using the other floral tissues (data not shown). To ensure that no artefact could arise during the course of the study, a similar dilution test was done for all the measurements described here. We also estimated the cAMP content of the floral tissues of three other lily varieties (*Lilium* cv. Apeldorn, *Lilium* cv. Star Gazer and *Lilium longiflorum* Thumb. cv. Nellie White). Although the flowers of the three cultivars were greatly dissimilar in size and colour, the cAMP content differed by no more than one order of magnitude between any of the tissues studied (data not shown).

The specificity of the RIA for the detection of cAMP was further confirmed by means of a phosphodiesterase control assay. Homogenates from upper parts of the style *Lilium* cv. Connecticut were treated with bovine spleen phosphodiesterase. As shown in Fig. 1, this treatment resulted in a loss of about 50% in detectable cAMP content compared with a control sample.

In animal tissues, cAMP is usually produced by an adenylyl cyclase transmembrane enzyme. Figure 2 shows that a similar activity is associated with the upper part (including stigma) of the plant style. This activity can be stimulated by forskolin, an activator of animal adenylyl cyclases and aluminium fluoride salts which activate G-protein, a typical regulator of animal adenylyl cyclases. The cAMP molecules detected (see Table 1) were thus most probably produced by a membrane-associated adenylyl cyclase which is under the control of a plant G-protein. The activation of membrane-bound adenylyl cyclase activity to detectable levels by forskolin has been observed previously in carrot cells [24]. However, the present study provides the first observation of a G-protein-mediated, aluminium fluoride stimulated pro-

Table 2. The cAMP content of various dilutions of an extract of the upper half of a style and a water extract*

	cAMP content (fmol/75 μl of diluted extract)	
Extract	Extract diluted by 4	Extract diluted by 8
Style (upper part) extract	16.0	10.1
Water extract	7.4	6.2
Specific tissue cAMP content	8.6	3.9

*An acetylated extract of the upper part of a style of *Lilium* cv. Connecticut or an acetylated water extract were obtained as described in the Experimental and were diluted four- or eight-fold. The cAMP content of 75 μl of the diluted extracts were then estimated by RIA and subtracted from one another to obtain the specific cAMP content of the tested tissue.

duction of adenylyl cyclase, even though GTP, a necessary substance for G-protein activity, has been clearly shown to be required for the detection of pea root membrane adenylyl cyclase activity [4].

The detection of significant quantities of cAMP in plant flower stigma and in other parts of the style suggests that cAMP may play a second messenger role in this tissue, as it does in animal cells. We thus applied various stresses to the lily flower to see whether they led to changes in cAMP concentration.

The first stress signal applied to the stigma was self-pollination. As shown in Fig. 3, this resulted in a 50% reduction in cAMP content in the top 1 cm of the flower style 30 min after self-pollination. Similar results have been observed in another lily flower cultivar (*Lilium* cv. Apeldorn; data not shown). The observed reduction in cAMP concentration in the flower style after self-pollination could, in fact, correspond to an inhibition of self-incompatible pollen tube growth which has been observed [17] to occur at about that same time. The same study also showed that forskolin is capable of reversing the inhibition of pollen tube elongation. Interestingly, forskolin also stimulated adenylyl cyclase activity in the upper part of the style of the lily flower (see Fig. 2). A comparison of the variation in the cAMP content observed in this organ after compatible and incompatible flower pollination is required to gain further understanding of this process.

Tepal wounding and flower emasculation are required during flower self-pollination. As can be seen in Fig. 3, these stresses are also capable of stimulating a reduction in the cAMP content (maximum at 90 min after wounding), but the time at which this occurs differs significantly from the reduction observed during self-pollination. The results of the pollination and wounding experiments shown in Fig. 3 were

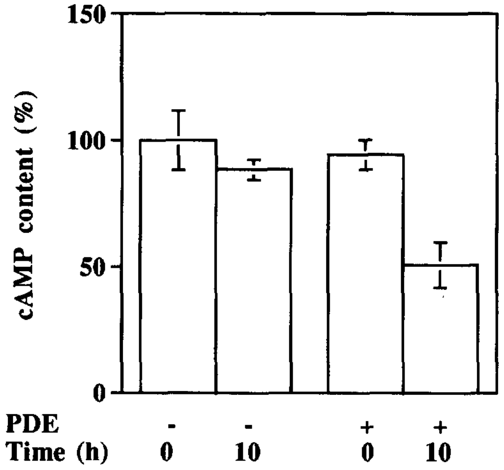

Fig. 1. Hydrolysis of plant cAMP by bovine spleen phosphodiesterase. Style upper parts, including stigma, of *Lilium* cv. Connecticut were homogenized and incubated for 0 or 10 hr in the presence (+PDE) or absence (−PDE) of phosphodiesterase II from bovine spleen. The results represent the quantity of cAMP present in each extract after each incubation period and are the mean ± standard error of three experiments. The quantity of cAMP present in the sample which did not contain any phosphodiesterase and was at time 0 was taken as 100%. For the 10 hr time point, $0.01 < P < 0.02$.

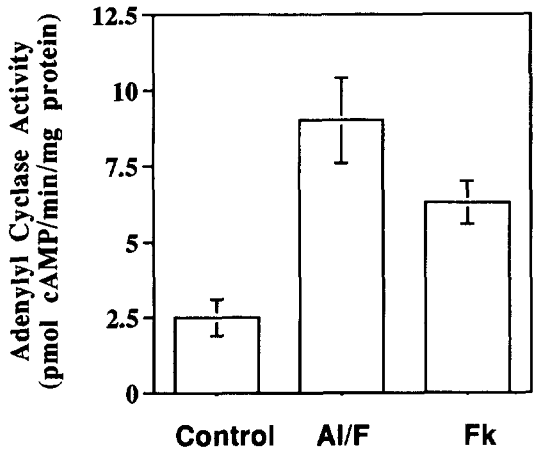

Fig. 2. Stimulation of adenylyl cyclase activity by AlF_4^- or forskolin. The adenylyl cyclase activity of a membrane fraction of style upper parts of *Lilium* cv. Connecticut was measured as described in the Experimental, without (control) and with 10 mM fluoroaluminate (Al/F) or 0.1 mM forskolin (Fk). The results are the mean ± standard error of three experiments. In both cases, $0.001 < P < 0.01$.

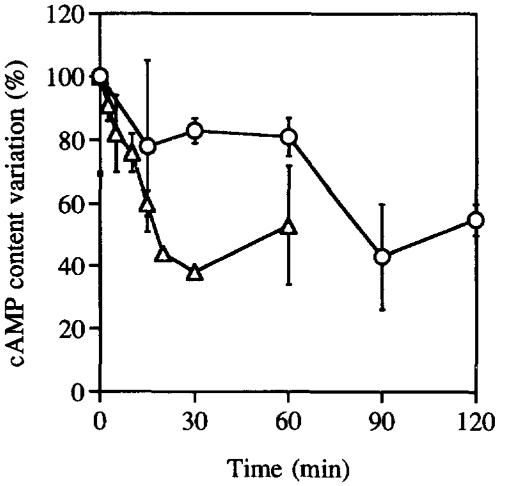

Fig. 3. Variations in the cAMP content of the upper part of the style after flower self-pollination or wounding. Flowers of *Lilium* cv. Connecticut were wounded by removing the upper half of the tepals and emasculation. They were then either pollinated with the pollen of a flower of the same cultivar (△), or left unpollinated (○). The cAMP content of the top 1 cm of the styles (including the stigma) was then estimated twice at varying times on two separate sets of flowers. Wounding and pollination experiments were done on both flower sets. The cAMP content at time zero was taken as 100% and all results are expressed as per cent variation from that value. Error bars correspond to S.E.M. values; they are sometimes smaller than the dots of some points and therefore do not appear on the graph.

obtained using the same batch of flowers to ensure that any difference in response speed did not reflect variations between two sets of flowers of the same species. Similar results and response times have been observed after wounding in two other lily cultivars (*Lilium* cv. Apeldorn and *Lilium* cv. Star Gazer; data not shown). This second stress-induced cAMP content change confirms that the cAMP detected is of plant and not microbial origin, as there is no simple link between a far distant signal and a response from micro-organisms which are anyway very rare in an unopened flower. The observation also suggests the existence of a long-distance signalling route between the tepals and the stigma of a flower. Such a long-distance signalling event was also seen in a recent study: enhanced kaempferol accumulation was observed in petunia stigmas after tepal removal [25]. Interestingly, enhanced kaempferol biosynthesis was also observed in this organ after flower pollination, just as we saw a lowering of the cAMP content during the self-pollination of lily flowers.

To the best of our knowledge, this is the first report of specific cAMP measurements in the floral organs of a plant. The observed levels are, however, about one order of magnitude lower than those in animal cells. For example, rat adipocyte cells contain 7.5 ± 0.1 pmol cAMP/mg protein [26], i.e. six times more than the level measured in the upper part of the style. The detection of cAMP in plant tissues can, however, still be specific and plant cAMP could play a second messenger function. It should be noted that plant stigma adenylyl cyclase activity is also about 10 times lower than that in adipocyte tissues [26]. Furthermore, plant cells are larger than animal cells, and thus represent a considerably more important cellular volume and endomembrane surface. If plant and animal cells were to express the same number of units of adenylyl cyclase activity per unit of plasma membrane surface, plant cells would display a lower adenylyl cyclase activity per milligram of endomembrane protein and a smaller quantity of cAMP per milligram of fresh weight of tissue.

EXPERIMENTAL

Obtaining flower tissues. Cut flowers of *L.* cv. Connecticut, *L.* cv. Apeldorn, *L.* cv. Star Gazer and *L. longiflorum* Thumb. cv. Nellie White were bought at a local flower shop and kept at 12°C until use. All flowers were treated and extracted at anthesis after the opening of the three external tepals and just before the opening of the three internal tepals. The flowers were cut swiftly into several parts with a sharp razor blade. The different parts assayed for cAMP were: one-third of a flower tepal, three filaments, three anthers, the ovaries, the top 1 cm of the style (including the stigma) and the lower part of the style. Dissected tissues were immediately ground in $HClO_4$ and the cAMP content measured, in order to avoid any unwanted cAMP hydrolysis or biosynthesis during tissue wounding.

Flower pollination and wounding. The flowers were wounded by decapitating their tepals at about half-height and mechanical removal of their anthers. The stigmas of the flowers were then either self-pollinated using the mature pollen of a more open flower on the same blooming branch (pollination experiment) or left unpollinated (wounding experiment) before being covered with a small piece of Al foil in order to prevent uncontrolled pollination. During the whole time course of the experiment, the flowers were left on their main blooming stem. At the desired time, the top 1 cm of the style (including stigma) was cut and immediately ground in $HClO_4$ for measurement of cAMP content.

cAMP measurement. Measurements were made according to the RIA method described in [27], with the following modifications. Tissues were homogenized into 650 μl of 1.1 M $HClO_4$ and centrifuged for 10 min at 15 000 *g* to separate the protein crystals from the acid-soluble cAMP. Protein and cAMP contents were then estimated using the same tissue extract as follows. Each pellet was resuspended into 500 μl of 10% TCA and sonicated at top speed for 2 min to allow the protein content to be quantified using an amido-black-based method [28]. A 300-μl aliquot of the supernatant of the tissue ground in $HClO_4$ was collected in parallel and mixed with 40 μl of 6 M K_2CO_3. The resulting ppt. was then removed by centrifugation (15 000 g for 10 min) and 150 μl of the supernatant was acetylated by the addition of 30 μl of 4 M KOH and 6 μl of Ac_2O. The acetylated supernatant was diluted four- to eight-fold and the cAMP content evaluated twice by RIA on 75-μl samples. The specific tissue cAMP content value was obtained by subtracting from the calculated value the value obtained on a similarly acetylated and diluted H_2O extract. The antibody calibration curve was obtained using the following cAMP concn (fmol in 75 μl): 1.5, 2.9, 5.9, 11.7, 23.4, 46.9, 93.8, 187, 375, 750, 1500.

Yield of cAMP extraction. The yield of the cAMP extraction was determined according to the following procedure. Styles (upper part) from *L.* cv. Connecticut were homogenized in 750 μl of 1.1 M $HClO_4$ containing 100 μl radio-iodinated cAMP (30 000 cpm) and centrifuged for 10 min at 15 000 g to separate the pellet from the acid-soluble cAMP. Radioactivity of an aliquot (100 μl) taken from the supernatant was determined in triplicate. Results were expressed as the mean ± standard error of three different experiments.

Phosphodiesterase assay. Styles (upper part) from *L.* cv. Connecticut were homogenized in 100 mM Tris–HCl, pH 6.8, containing 0.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$. Half the homogenate was incubated with 2 units of phosphodiesterase II (EC 3.1.16.1) from bovine spleen at pH 6.8 for 10 hr at 37°; the second half of the homogenate received the same treatment except that the hydrolytic enzyme was omitted. Assayswere stopped with $HClO_4$ (1 M final concn) and the cAMP content determined using the RIA described above.

Adenylyl cyclase activity. Adenylyl cyclase was assayed using membrane fractions in the presence of phosphodiesterase inhibitor according to [29]. Stigma membranes were obtained after homogenization in a glass Potter of 1.5 g of the upper part of the styles of flowers of *L.* cv. Connecticut in 1 ml of 40 mM Tris/HCl, pH 7.4, containing 250 mM sucrose, 40 μg ml^{-1} leupeptin and aprotinin, 0.1 mM phenylmethysulphonyl fluoride and 0.1 mM dithiothreitol (buffer A). The homogenate was subjected to differential centrifugation (20 000 g for 10 min followed by 100 000 g for 80 min) and the last pellet resuspended into 100 μl of buffer A. Incubations were performed in the presence of various stimulators (10 mM AlF_4^- or 0.1 mM forskolin) for 15 min at 30° in 100 μl 40 mM Tris–HCl, pH 7.4, containing 10–20 μg membrane protein with 0.4 mM [α-^{32}P] ATP (10^5 cpm/assay; Du-Pont, New England Nuclear) 4 mM $MgCl_2$, 1 mM RO 7-2956, 0.5 U/ml adenosine deaminase and an ATP-regenerating system [30]. AlF_4^-, the active stimulator, was obtained *in situ* by mixing $AlCl_3$ and NaF solutions at the beginning of each experiment. Assays were carried out in triplicate and the protein content of the samples estimated according to [28].

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REFERENCES

1. Rall, T. W., Sutherland, E. W. and Berthet, J., *Journal of Biological Chemistry*, 1957, **224**, 463.
2. Pastan, I. and Perlman, R. L., *Advances in Cyc. Research*, 1972, **1**, 11.
3. Assmann, S. M., *Plant Physiology*, 1995, **108**, 885.
4. Pacini, B., Petrigliano, A., Diffley, P., Paffetti, A., Brown, E. G., Martinelli, P., Trabalzini, L., Bovalini, L., Lusini, P. and Newton, R. P., *Phytochemistry*, 1993, **34**, 899.
5. Gromes, R. and Zetsche, K., *Botanica Acta*, 1992, **105**, 395.
6. Al-Azzawi, M. J. and Hall, J. L., *Plant Science Letters*, 1976, **6**, 285.
7. Brown, E. G., Al-Najafi, T. and Newton, R. P., *Phytochemistry*, 1977, **16**, 1333.
8. Gangwani, L., Khurana, J. P. and Maheshwari, S. C., *Phytochemistry*, 1994, **35**, 857.
9. Komatsu, S. and Hirano, H., *Plant Science*, 1993, **94**, 127.
10. Baizabal-Aguirre, V. M. and Gonzalez de la Vera, L. E., *Physiologia Plantarum*, 1994, **91**, 147.
11. Newton, R. P. and Brown, E. G., in *Hormones, Receptors and Cellular Interactions in Plants*, eds C. M. Chadwick and D. R. Garrod. Cambridge University Press, Cambridge, 1986, pp. 115–153.
12. Cooke, C. J., Smith, C. J., Walton, T. J. and Newton, R. P., *Phytochemistry*, 1994, **35**, 889.
13. Kurosaki, F., Tsurusawa, Y. and Nishi, A., *Phytochemistry*, 1987, **26**, 1919.
14. Curvetto, N. and Delmastro, S., *Plant Physiology and Biochemistry*, 1990, **28**, 367.
15. Chung, C. H., Lim, S. U., Song, P. S., Berlin, J. D. and Goodin, J. R., *Plant Cell Physiology*, 1988, **29**, 855.
16. Khurana, J. P., Tamot, B. K. and Maheshwari, S. C., *Plant Cell Physiology*, 1988, **29**, 1023.
17. Tezuka, T., Hiratsuka, S. and Takahashi, S. Y., *Plant Cell Physiology*, 1993, **34**, 955.
18. Li, W., Luan, S., Schreiber, S. L. and Assmann, S. M., *Plant Physiology*, 1994, **106**, 957.
19. Spiteri, A., Viratelle, O. M., Raymond, P., Rancillac, M., Labouesse, J. and Pradet, A., *Plant Physiology*, 1989, **91**, 624.
20. Newton, R. P., Gibbs, N., Moyse, C. D., Wiebers, J. C. and Brown, E. G., *Phytochemistry*, 1980, **19**, 1909.
21. Ishioka, N. and Tanimoto, S., *Plant Cell Physiology*, 1990, **31**, 91.
22. Bolwell, G. P., *Phytochemistry*, 1992, **31**, 4081.
23. Gangwani, L., Tamot, B. K., Khurana, J. P. and Maneshwari, S. C., *Biochemical and Biophysical Research Communications*, 1991, **178**, 1113.
24. Kurosaki, F. and Nishi, A., *Archives of Biochemistry and Biophysics*, 1993, **302**, 144.
25. Vogt, T., Pollak, P., Tarlyn, N. and Taylor, L. P., *The Plant Cell*, 1994, **6**, 11.
26. Dib, K., Oget, I., Wrisez, F., El Jamali, A., Aguie-Aguie, G., Corrèze, C. and Lambert, B., *International Journal of Obesity*, 1996, **20**, 15.

27. Cailla, H. L., Racine, L., Weisbuch, M. S. and Delaage, M. A., *Analytical Biochemistry*, 1973, **56**, 394.
28. Schaffner, W. and Weissmann, C., *Analytical Biochemistry*, 1973, **56**, 502.
29. Salomon, Y., Londos, C. and Rodbell, M., *Analytical Biochemistry*, 1974, **58**, 541.
30. Saunier, B., Dib, K., Delemer, B., Jacquemin, C. and Corrèze, C., *Journal of Biological Chemistry*, 1990, **265**, 19942.