EFFECTS OF ARACHIDONATE ON CULTURED PIG THYROID CELLS AND THEIR STIMULATION BY THYROTROPIN

Corinne GERARD, Bernard HAYE and Claude JACQUEMIN
Laboratoire de Biochimie, UER Sciences, BP 347, 51062 Reims cedex, France

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1. Introduction

Arachidonate has been reported to interfere in complex fashion with the metabolism of the dog thyroid gland [1]. As in numerous other tissues arachidonate stimulates prostaglandin synthesis but with a low conversion rate. Moreover, in thyroid it depresses the accumulation of cyclic AMP induced by TSH. Arachidonate inhibits the thyroid secretion stimulated by low concentrations of TSH but enhances the iodination of proteins in dog thyroid slices. These effects do not depend on prostaglandin conversion, the decrease in the stimulation of adenylate cyclase is likely to be due to the unsaturated character of the fatty acid but it has been suggested that the stimulation of thyroid protein iodination is a consequence of an increase in lipoxygenase activity. Our purpose was to extend these studies further to cultured pig thyroid cells in various physiological controled states and to draw a particular attention to phospholipid metabolism, protein iodination and cyclic AMP stimulation.

The results obtained show that the mode of action of arachidonate is more complex than described in [1] and affects the adenylate cyclase system but also the iodinating system and the phospholipid metabolism.

2. Materials and methods

2.1. *Preparation and culture of thyroid cells*

Thyroid cells were isolated from pig glands by a discontinuous trypsinization technique [2]. Freshly isolated cells, suspended in Eagle's minimum essential medium (pH 7.4) with 10% (v/v) calf serum, penicillin (200 units/ml) and streptomycin sulfate (50 μg/ml) were cultured, at $2-3 \times 10^6$ cells/ml in 100 mm diam. plastic Petri dishes (Falcon) not treated for tissue culture at 37°C in 95% air–5% CO_2 water-satured atmosphere with or without TSH.

2.2. *Cell washing*

After 1–4 days in culture, cells were centrifuged at 200 × g for 7 min. The pellet was resuspended in Earle's solution buffered with 20 mM hepes (pH 7.2) and the cells were centrifuged again. This washing procedure was repeated 2 times. After the last washing, the cells were suspended in Earle's–Hepes buffer (pH 7.2).

2.3. *Cyclic AMP assay*

Aliquots (400 μl, 0.1–0.3 mg protein) of washed thyroid cell suspension were air-incubated for 5 min at 37°C in a final volume of 700 μl containing Earle's–Hepes buffer (pH 7.2), isobutylmethylxanthine (IBMX) (10^{-3} M) and with or without TSH (40 mU/ml). The incubation was terminated by addition of 78 μl 10 N $HClO_4$ and immersion of the tubes into an ice bath. The cells were homogenized and the cyclic AMP content was assayed by the radioimmunological method in [3] except that bound and free ligand were separated by precipitation of bound ligand with a mixture of γ-globulin (2.5 mg/ml in citrate buffer (pH 6.2)) and polyethylene glycol 6000 (20 g in 100 ml water).

2.4. *Phospholipid assay*

Aliquots (500 μl, 0.1–0.3 mg protein) of washed thyroid cell suspension were air-incubated for 2 h at 37°C in final volume of 700 μl containing Earle's–Hepes buffer (pH 7.2), [^{32}P]orthophosphate (10 μCi). The incubation was terminated by addition of a chloroform–methanol mixture (2:1, v/v). The phospholipids were extracted and analyzed as in [4]. The (PI cpm/mg protein)/(PC cpm/mg protein) ratio is calculated as an index of the 'phospholipid effect'.

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Table 1

Effect of arachidonate and TSH on the protein iodination of control cultured cells and TSH treated cells

Acute treatment \ Chronic treatment		One day control cells		One day TSH-treated cells (0.1 mU/ml)		Four day control cells		Four day TSH-treated cells (0.1 mU/ml)	
TSH (40 mU/ml)	Arachidonate 0.1 mM	$PB^{125}I$ cpm/mg protein	cAMP pmol/mg protein	$PB^{125}I$ cpm/mg protein	cAMP pmol/mg protein	$PB^{125}I$ cpm/mg protein	cAMP pmol/mg protein	$PB^{125}I$ cpm/mg protein	cAMP pmol/mg protein
–	–	51 847 ± 1327	32 ± 1	100 297 ± 645	45 ± 2	16 739 ± 139	17 ± 1	1 932 509 ± 18 674	27 ± 1
–	+	136 088 ± 1659	29 ± 1	127 329 ± 608	51 ± 3	53 611 ± 2084	32 ± 1	286 736 ± 22 295	71 ± 11
+	–	67 233 ± 1721	596 ± 7	410 909 ± 3986	412 ± 11	16 181 ± 411	104 ± 1	2 379 443 ± 11 919	699 ± 17
+	+	146 201 ± 2454	232 ± 6	257 578 ± 2900	300 ± 5	47 681 ± 33	68 ± 4	266 787 ± 29 567	642 ± 54

After washing, the cells were incubated 45 min, a 37°C in Earle's–Hepes buffer (pH 7.2) in final volume of 700 μl containing 1 μCi Na ^{125}I. At the end of the incubation, 1.3 ml Earle's–Hepes containing KI (10^{-4} M) and bovine serum albumin fraction V (5 mg/ml) were added and 2 ml cold 20% trichloroacetic acid. After centrifugation (500 × g, for 5 min) the pellet was resuspended and washed with cold 10% trichloroacetic acid (2 times) then the pellet was counted as $PB^{125}I$

2.5. *Thyroid proteins iodination*

The evaluation of the thyroid proteins iodination by the incorporation of radioiodine in the trichloroacetic acid precipitate of the cells, were performed as described in the legend of table 1.

2.6. *Other methods and chemicals*

Protein estimation was performed according to [5] using bovine serum albumin in 0.1 M NaOH as standard. Aliquots of cells were centrifuged and the pellets solubilized in 0.1 N NaOH. Bovine TSH 3.5 U/mg (NIH, TSH B_8) was a gift of the NIH (Bethesda MD), [^{32}P]orthophosphate (10 mCi/ml) and Na ^{125}I (2 mCi/ml) were purchased from the CEN Saclay, trypsin from Grand Island Biological Co. (Grand Island NY), newborn calf serum from Flow Laboratories (Irvine), minimum essential medium from Merieux (Lyon), dibutyrylcyclic adenosine monophosphate from Calbiochem, prostaglandin E_2 was a gift of Dr Pike (Upjohn Co.). Arachidonic acid was purchased from Applied Science Labs. Indomethacin was a gift from Merck, Sharp and Dohme.

2.7. *Statistical evaluation*

The statistical differences were calculated using Student's t-test: the mean values of triplicate determinations were expressed with standard errors of the mean (SEM).

Experiments were reproduced 2–3-times with very similar or identical results. Typical experiments are described in tables and figures.

3. Results

3.1. *Cyclic AMP*

3.1.1. One day culture

The time course of the effect of arachidonate in the presence of indomethacin on the cyclic AMP responsiveness to TSH (40 mU/ml) of control cells is shown in fig.1. The same results are obtained in the absence of indomethacin (20 μg/ml).

The inhibition is very rapid in onset when the cells are stimulated by TSH and increases with the arachi-

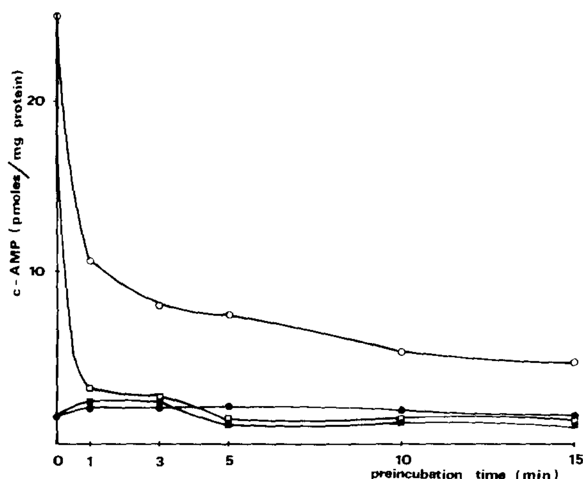

Fig.1. Time course of the effect of arachidonate on the cyclic AMP responsiveness to TSH by one day cultured control cells. After washing (see section 2), the cells were preincubated at 37°C in Earle's–Hepes buffer (pH 7.2) in presence of arachidonate and indomethacin (20 μg/ml). After the indicated preincubation time, TSH (40 mU/ml) and IBMX (10^{-3} M final conc.) were added in a total volume of 700 μl. The incubation was pursued 5 min, stopped with $HClO_4$ 10 N and the cyclic AMP content determined: (•) control cells + preincubation in presence of arachidonate 0.1 mM and indomethacin + 5 min incubation in presence of IBMX without TSH; (○) control cells + preincubation in presence of arachidonate 0.1 mM and indomethacin + 5 min incubation in presence of TSH and IBMX; (■) control cells + preincubation in presence of arachidonate 1 mM and indomethacin + 5 min incubation in presence of IBMX without TSH; (□) control cells + preincubation in presence of arachidonate 1 mM and indomethacin + 5 min incubation in presence of IBMX and TSH.

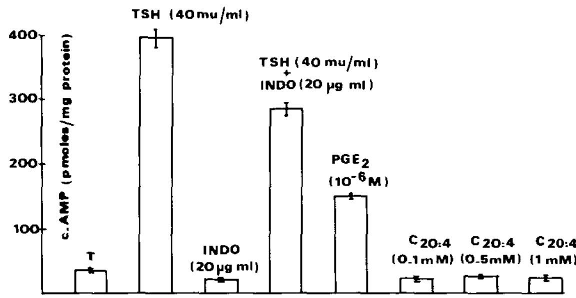

Fig.2. Effect of TSH, PGE_2, arachidonate (C20:4) and indomethacin on the cyclic AMP accumulation of 1 day cultured control cells. After washings the cells were incubated 5 min at 37°C in Earle's–Hepes (pH 7.2) in presence of the different effectors and IBMX (10^{-3} M).

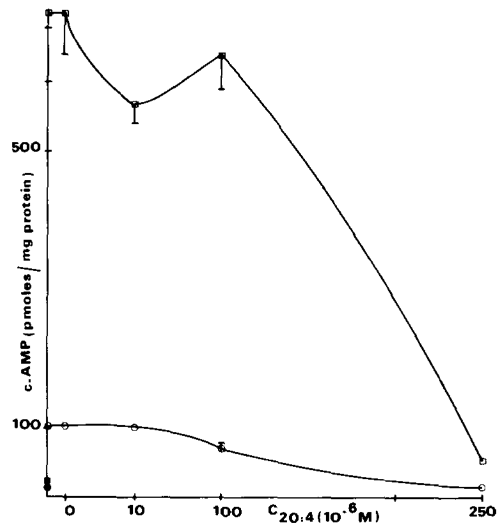

Fig.3. Effect of arachidonate on the cyclic AMP responsiveness to TSH of 4 day cultured control cells and TSH (0.1 mU/ml) treated cells. After washings, the cells were preincubated 15 min 37°C in Earle's–Hepes in presence of different concentrations of arachidonate without indomethacin. Then the incubation was pursued 5 min in presence of TSH (40 mU/ml) and IBMX (10^{-3} M): (●) control cells; (○) control cells + TSH (40 mU/ml); (■) TSH-treated cells; (□) TSH-treated cells + TSH (40 mU/ml).

donate concentration: 60% in 1 min at 0.1 mM and 95% in 1 min at 1 mM.

The stimulation by PGE_2 (10^{-6} M) of the cyclic AMP response of the 1 day control cells demonstrates the presence of PG receptors in these cells (fig.2).

Arachidonate does not stimulate the cyclic AMP system of these cells but indomethacin partially inhibits the TSH stimulation of the cyclic AMP as we already described in pig thyroid slices [6] (fig.2).

3.1.2. Four day culture

The age of the culture and the physiological state of the cells influence the TSH response [7] and the arachidonate effect.

The inhibitory character of arachidonate on the cyclic AMP responsiveness to TSH (40 mU/ml) decreases when the cells were cultured in the presence of TSH (0.1 mU/ml). The total inhibition of the TSH responsiveness is obtained with a 15 min preincubation in the presence of 0.25 mM arachidonate (fig.3).

On the same cells, arachidonate does not stimulate or very slightly stimulates the cyclic AMP system of control or TSH-treated cells (0.1 mU/ml). Indomethacin does not inhibit the TSH stimulation (40 mU/ml) of the control cells (172 ± 9 pmol cAMP/mg protein without indomethacin; 167 ± 1 pmol cAMP/mg protein with indomethacin; control, 50 ± 1 pmol cAMP/mg protein) but partially inhibits the TSH stimulation of the TSH-treated cells (667 ± 20 pmol cAMP/mg protein without indomethacin; 528 ± 14 pmol cAMP/mg protein with indomethacin; control, 50 ± 5 pmol cAMP/mg protein).

3.2. *Protein iodination*

3.2.1. One day culture

On control cells at day 1, arachidonate stimulates protein iodination in a similar manner whether TSH (40 mU/ml) is present or not (table 1). The situation is inverted when TSH (0.1 mU/ml) was present during the culture. The chronic treatment stimulates the ^{125}I incorporation into proteins by 100%. Arachidonate slightly active by itself inhibits by 50% the 4-fold increase in iodination promoted by TSH (40 mU/ml).

3.2.2. Four day culture

The basal ^{125}I incorporation into proteins of the 4 days cultured control cells is smaller than the corresponding values at day 1. TSH (40 mU/ml) is without effect on these control cells. Arachidonate stimulates the iodination in these cells in the presence or absence of TSH (40 mU/ml). The chronic treatment of the cells with TSH (0.1 mU/ml) increases the iodination level 19 times and the acute stimulation with TSH (40 mU/ml) raises it only by 23%. Under these conditions arachidonate is dramatically inhibitory, the pro-

Table 2
Effect of arachidonate and TSH on the phospholipid metabolism of control cultured cells and TSH-treated cells

Acute treatment	Chronic treatment	One day control cells		One day TSH-treated cells (0.1 mU/ml)		Four day control cells		Four day TSH-treated cells (0.1 mU/ml)	
TSH 40 mU/ml	Arachidonate 0.1 mM	Phospholipid ^{32}P incorp. (cpm/mg protein)	cAMP pmol/mg protein	Phospholipid ^{32}P incorp. (cpm/mg protein)	cAMP pmol/mg protein	Phospholipid ^{32}P incorp. (cpm/mg protein)	cAMP pmol/mg protein	Phospholipid ^{32}P incorp. (cpm/mg protein)	cAMP pmol/mg protein
		PLT 13 453 ± 579		PLT 11 432 ± 219		PLT 7 839 ± 30		PLT 15 760 ± 346	
–	–	PI 6 476 ± 488	32 ± 1	PI 6 597 ± 151	45 ± 2	PI 2 184 ± 79	17 ± 1	PI 7 192 ± 223	27 ± 1
		PC 4 353 ± 101		PC 2 786 ± 66		PC 2 406 ± 23		PC 4 486 ± 150	
		PI/PC 1.47		PI/PC 2.37		PI/PC 0.91		PI/PC 1.60	
		PLT 15 405 ± 1010		PLT 18 906 ± 366		PLT 6 352 ± 615		PLT 9 480 ± 208	
–	+	PI 6 443 ± 500	29 ± 1	PI 10 027 ± 230	51 ± 3	PI 2 057 ± 301	32 ± 1	PI 2 437 ± 604	71 ± 11
		PC 6 844 ± 347		PC 6 533 ± 112		PC 1 766 ± 196		PC 5 012 ± 1133	
		PI/PC 0.94		PI/PC 1.53		PI/PC 1.15		PI/PC 0.48	
		PLT 14 076 ± 47		PLT 12 621 ± 260		PLT 11 212 ± 814		PLT 19 914 ± 126	
+	–	PI 6 261 ± 72	596 + 7	PI 7 031 ± 195	412 ± 11	PI 4 363 ± 201	104 ± 1	PI 8 868 ± 180	699 ± 17
		PC 4 884 ± 41		PC 3 007 ± 86		PC 3 103 ± 188		PC 6 606 ± 48	
		PI/PC 1.28		PI/PC 2.34		PI/PC 1.41		PI/PC 1.34	
		PLT 17 196 ± 79		PLT 20 439 ± 365		PLT 7 795 ± 446		PLT 10 672 ± 1360	
+	+	PI 7 899 ± 134	232 ± 6	PI 10 853 ± 368	300 ± 5	PI 2 952 ± 364	68 ± 4	PI 2 764 ± 549	642 ± 54
		PC 6 604 ± 28		PC 6 444 ± 148		PC 2 008 ± 157		PC 5 413 ± 648	
		PI/PC 1.19		PI/PC 1.68		PI/PC 1.45		PI/PC 0.51	

After washing, the cells were incubated for 2 h at 37°C in Earle's–Hepes buffer (pH 7.2) in presence of [^{32}P]orthophosphate (10 μCi) with or without TSH (40 mU/ml. The phospholipids were extracted and analyzed as in [4]: PLT, total phospholipids; PI, phosphatidylinositol; PC, phosphatidylcholine

tein-bound iodine decreases by 85% in the absence and by 88% in the presence of TSH (40 mU/ml).

On the same cells, the corresponding values of the cyclic AMP content are reported in table 1.

3.3. ^{32}P *incorporation into phospholipids*

3.3.1. One day culture

The cell phospholipid labelling is greatly affected by culture conditions. The general incorporation of [^{32}P]orthophosphate in the phospholipids of the control cells decreases with the duration of the culture (table 2).

Arachidonate increases the general labelling of phospholipids at day 1 of control or TSH-treated cells in the presence or absence of TSH (40 mU/ml) in the incubation media.

All these general modifications are a reflexion of individual changes in the labelling of the various phospholipids. The 'normal phospholipid effect' (increase of PI/PC labelling ratio) produced in thyroid slices by an acute TSH stimulation is decreased or even disappears in control cell cultures.

With chronically TSH treated cells a new steady-state of labelling, corresponding to the stimulated state (high PI/PC ratio) is reached. Then, an acute TSH treatment produces a 'reverse phospholipid effect' (decrease of the PI/PC labelling ratio) (submitted).

On control or TSH-treated cells, arachidonate has a 'reverse effect' on the PI/PC ratio. When TSH and arachidonate are present together in the acute incubations, the acute TSH effect, if any, is almost masked by that of arachidonate on control or TSH-treated cells (table 2).

3.3.2. Four day culture

Arachidonate decreases the general labelling of phospholipids at day 4. Arachidonate has no effect at day 4 on the PI/PC ratio of control cells but has a very strong 'reverse effect' on the TSH-treated cells (table 2).

When TSH and arachidonate are present together in the acute incubations the very strong 'reverse effect' on the TSH-treated cells still exists.

On the same cells, the corresponding values of the cyclic AMP content are reported in table 2. The relationships between the ^{32}P-incorporation into phospholipids and the stimulation of the cyclic AMP are discussed elsewhere (submitted).

4. Discussion

The effects of arachidonate on thyroid metabolism and its stimulation by TSH that we observed with cultured pig thyroid cells are not as simple to interpret as it appears in [1] with dog thyroid slices.

We confirmed in our system the inhibition by arachidonate of the cyclic AMP accumulation induced by TSH but this inhibition is less effective on 4 day TSH-treated cells (0.1 mU/ml).

We confirmed in our system the stimulatory effect of arachidonate on protein iodination on 1 day cultured control cells but other results show that the mode of action of arachidonate on thyroid is more complex and depends on the physiological state of the cells:

(1) The inhibitory effect of arachidonate (0.1 mM) upon the TSH stimulation of adenylate cyclase decreased with the ageing of the culture; more so as TSH (0.1 mU/ml) was present in the culture.

Under this condition, at day 4 in the reported experiment, the stimulation of cyclic AMP accumulation by TSH (40 mU/ml) was maximum (25-fold) and there was no more effect of 0.1 mM arachidonate. Nevertheless, a very strong inhibition was obtained with 0.25 mM arachidonate. We confirmed that this effect was inhibited neither by indomethacin nor by Etya (not shown), so it appeared that the metabolites of arachidonate were not involved in this inhibition.

In our system, exogenous arachidonate does not stimulate or very slightly stimulates the cyclic AMP system in a 15 min experiment. On the same system it was found [8] that control cultured pig thyroid cells accumulated 6–10-times more PGE_2 than TSH-treated cells. The depressive effect of TSH they observed on the PG synthetase activity was in contrast with that obtained [9] on the rat thyroid PG synthetase activity after in vivo treatment.

In our system, the cyclic AMP responsiveness to TSH in the presence of indomethacin shows that the PG synthetase activity was greatly affected by the culture conditions. Indomethacin diminished the cyclic AMP responsiveness to TSH of 1 day cultured control cells (33%) but not of 4 day cultured control cells. On the TSH-treated cells (0.1 mU/ml), indomethacin inhibited the cyclic AMP responsiveness to TSH of 1 day and 4 day cultured cells by 18% and 21%, respectively.

So, with cultured pig thyroid cells, the interpretation is quite different if we consider the basal PG syn-

thetase activity or the PG synthetase stimulation potential by an 'acute' high concentration of TSH.

(2) We confirmed in our system the stimulation by arachidonate of the thyroid proteins iodination of 1 day or 4 day cultured control cells.

However, we obtained an inhibition by arachidonate of the iodination level when TSH (0.1 mU/ml) was present during the culture or when TSH (40 mU/ml) was present during the incubation. Arachidonate (0.1 mM) was dramatically inhibitory on 4 day TSH-treated cells iodination. Our results show that there is no direct correlation between the iodination of the proteins and the stimulation of the cyclic AMP. Indeed, we observed that when the cells were submitted to acute TSH stimulation, protein iodination was more sensitive to arachidonate inhibition than cyclic AMP level. In some experiments the former was strongly inhibited whereas the latter is not affected. We suggest two modes of action of arachidonate:

(i) That related to the unsaturated character of the fatty acid affecting the adenylate cyclase stimulation and the subsequent cAMP dependent events;

(ii) That more distal and interacting with the iodinating system.

'Peroxidases could provide a new pathway of arachidonate metabolism besides cyclooxygenase and lipoxygenases' [10]; our results are compatible with this hypothesis. In [11] we demonstrated a lack of correlation between the increase in the free arachidonate pool and its incorporation into prostaglandins. In [12,13] our results [6] on the PG production under an acute TSH stimulation of the phosphatidylinositol (PI) metabolism were confirmed. Specific PI phospholipases demonstrated and characterized in pig thyroid plasma membranes [12,13]. So the arachidonate liberated by the hormone action:

1. Is a substrate for cycloxygenase and lipoxygenase;
2. Could modulate the thyroid activity by interacting directly with the TSH receptors or adenylate cyclase system;
3. Could modulate the thyroid activity after reacylation of plasma membrane phospholipids or modification of the membrane phospholipids metabolism as described here;
4. Could modulate the iodination process via a new arachidonate metabolite [10].

Here we show that the arachidonate control of the iodination process can be positive or negative varying with the physiological state of the thyroid, and is not in direct relation with cyclic AMP system stimulation.

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References

[1] Boeynaems, J. M., Van Sande, J., Decoster, C. and Dumont, J. E. (1980) Prostaglandins 19, 537–550.
[2] Mauchamp, J., Margotat, A., Chambard, M., Charrier, B., Remy, L. and Bechet, M. (1979) Cell. Tiss. Res. 204, 417–430.
[3] Cailla, H. L., Racine-Weisbuch, M. S. and Delaage, M. A. (1973) Anal. Biochem. 56, 394–407.
[4] Jacquemin, C. and Haye, B. (1970) Bull. Soc. Chim. Biol. 52, 153–165.
[5] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[6] Haye, B., Champion, S. and Jacquemin, C. (1973) FEBS Lett. 30, 253–260.
[7] Takasu, N., Charrier, B., Mauchamp, J. and Lissitzky, S. (1978) Eur. J. Biochem. 90, 139–146.
[8] Margotat, A., Rolland, P. H., Charrier, B. and Mauchamp, J. (1978) FEBS Lett. 95, 347–351.
[9] Levasseur, S., Friedman, Y. and Burke, G. (1980) Endocrinology 107, 2041–2044.
[10] Boeynaems, J. M. and Hubbard, W. C. (1980) J. Biol. Chem. 255, 9001–9004.
[11] Haye, B. and Jacquemin, C. (1977) Biochim. Biophys. Acta 487, 231–242.
[12] Igarashi, Y. and Kondo, Y. (1980) Biochem. Biophys. Res. Commun. 97, 759–765.
[13] Igarashi, Y. and Kondo, Y. (1980) Biochem. Biophys. Res. Commun. 97, 766–771.