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INDUCTION OF STEROID-BINDING ACTIVITY IN *PSEUDOMONAS TESTOSTERONI*

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SUMMARY

Growth of *Pseudomonas testosteroni* on testosterone was optimal at 30°C. Induction of steroid-binding activity occurred optimally at 25°C to 30°C. Binding activity first appeared after 9 h of growth on testosterone and reached its maximum at 30 h. Dehydrogenation activities, on the other hand, reached a maximum at 12 h. Steroid-binding activity was induced by growth on several C19 and C21 steroids. At incubation temperatures of 37°C or 45°C there was minimal induction of binding activity. Binding activity induced at 30°C was irreversibly destroyed at 45°C and reversibly inhibited at 37°C.

INTRODUCTION

Pseudomonas testosteroni is an organism capable of adaptive growth on testosterone and other steroids[1–3]. A steroid receptor induced during growth can be released by exposure of induced cultures to osmotic shock[4]. The receptor demonstrated characteristics of a protein molecule, was saturable at low hormone concentrations, and had a high affinity for steroids. The exact role of the receptor protein is under investigation but, as with other bacterial periplasmic proteins, it is postulated to be involved in some aspect of steroid transport through the cell membrane. In this report we describe the conditions for induction of this steroid receptor and the factors which must be controlled in demonstrating steroid binding activity.

EXPERIMENTAL PROCEDURES

Materials

P. testosteroni 11996 was obtained from American Type Culture Collection, Rockville, Maryland; [1,2-^{3}H]-testosterone (45 Ci/mmol), [7-^{3}H]-testosterone (25 Ci/mmol), [1,2-^{3}H]-androstenedione (48 Ci/mmol), [1,2-^{3}H]-aldosterone (50 Ci/mmol), [1,2-^{3}H]-cortisol (43 Ci/mmol), [1,2-^{3}H]-progesterone (50·3 Ci/mmol), 17α-[1,2-^{3}H]-hydroxyprogesterone (49·2 Ci/mmol) and Aquasol from New England Nuclear Corporation, Boston, Massachusetts; unlabeled steroids from Mann Research Laboratories, New York, New York, and Steraloids, Inc., Pawling, New York; and Dextran T70 from Pharmacia, Uppsala, Sweden.

Methods

Growth conditions, induction of binding activity. Media used for growth and conditions for induction of binding activity have been described[4]. All steroids were added to a final concentration of 0·5 g/l for induction of binding activity.

Assay for binding activity. The binding activity of bacterial cells was assayed as described [4].

Assay for dehydrogenation activity of bacterial cells. Reaction mixtures for assay of dehydrogenation activities contained in 1 ml: 50 mM Tris-HCl, pH 9·0, [1,2-^{3}H]- or [7-^{3}H]-testosterone of known specific activity, and appropriate volumes of induced or uninduced bacteria.

Dehydrogenation activity was usually assayed for 10 min at 30°C. The reaction was terminated by the addition of 0·3 ml of dextran-coated charcoal (2·5% neutral Norit-0·25% Dextran T70), and the bacteria and dextran-coated charcoal were removed by centrifugation at 3,000 *g* for 10 min at 4°C. The supernatant was decanted and 0·5 ml was added to 10 ml of Aquasol. Radioactivity was determined in a liquid scintillation spectrometer. The radioactivity in the supernatant after charcoal adsorption was analyzed in several experiments and found to be totally ^3H_2O. In subsequent experiments, therefore, ^3H_2O was assayed as non-charcoal adsorbable material remaining in the supernatant. Tritiated water was determined by a method described by Vaughan and Boling [5]. One unit of dehydrogenation activity was defined as 1 pmol of labeled steroid dehydrogenated under the conditions of the experiment.

RESULTS

Effect of temperature on induction of steroid-binding activity and bacterial growth on testosterone

The optimum temperature for growth on testosterone of *P. testosteroni* was investigated. Optimum growth occurred at 30°C (Table 1). The organism was able to grow at 25°C, but very little growth occurred at 37°C and none at 45°C.

When the effect of incubation temperatures on induction of steroid-binding activity was investigated, cultures grown at 25°C and 30°C had the greatest binding activity when cultures were assayed 30 h after induction (Table 2). Cultures grown at 25°C had slightly greater binding activity than cultures grown at 30°C and this may be related to the more rapid utilization of steroid substrate at 30°C than at 25°C. Cultures grown at 37°C had very little binding activity whereas cultures induced at 45°C demonstrated no binding activity.

Inhibition of binding activity at 37°C and 45°C

The inability to demonstrate binding activity at 37 and 45°C could be related to the thermolability of the binding protein. The binding activity of cultures induced at 30°C was assayed at 37 and 45°C. There was a rapid loss of binding activity at these temperatures which was evident by 5 min of incubation (Fig. 1). In order to

Table 1. Growth of *P. testosteroni* on testosterone: effect of incubation temperatures

Incubation temperature	Absorbance (λ_{660}) (induced/uninduced after 30 h)
45°C	1·00
37°C	1·11
30°C	1·89
25°C	1·26

Table 2. Induction of binding activity: effect of incubation temperatures

Temperature during induction	Binding activity (pmoles/ml bacteria)
45°C	0
37°C	16·6
30°C	100·8
25°C	128·0

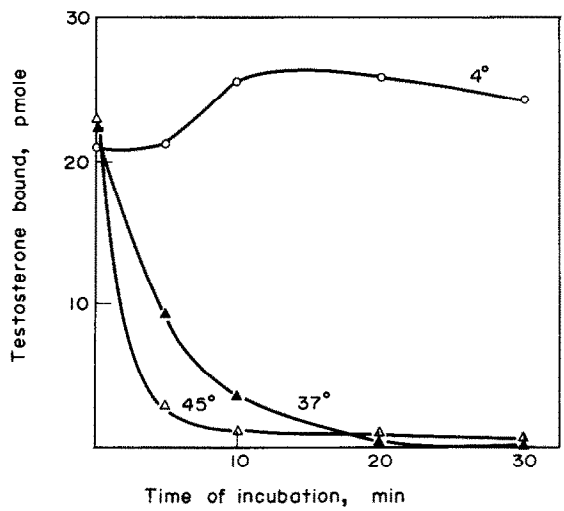

Fig. 1. Effect of temperature on binding activity. The reaction mixture, containing 0·3 ml of bacteria induced at 30°C and 80 pmol of [7-^{3}H]-testosterone ($2{\cdot}7 \times 10^6$ d.p.m./nmol) was incubated at 4, 37 or 45°C for the time periods indicated. Binding activity was assayed as described [4].

examine the reversiblility of the inhibition at these temperatures, cells induced at 30°C were pre-incubated at 30, 37 and 45°C for periods up to 30 min, then rapidly cooled to 4°C and assayed for binding activity at this temperature. Cells pre-incubated at 37°C were capable of binding testosterone when subsequently restored to 4°C indicating that the temperature effect was reversible (Fig. 2). Cells pre-incubated at 45°C, on the other hand, demonstrated a rapid irreversible loss of binding activity.

Induction and steroid-binding activity with various C19 and C21 steroids

Steroids other than testosterone are known to support growth of this organism [6]. Binding activity was assayed on bacteria grown on some of these C19 and C21 steroids. In addition to testosterone, androstenedione, androsterone, 5α- and 5β-dihydrotestosterone, dehydroepiandrosterone and progesterone induced significant binding activity (Table 3).

Cultures induced by testosterone were examined for their ability to bind other steroids. Both testosterone and androstenedione were bound (Table 4). Of the C21

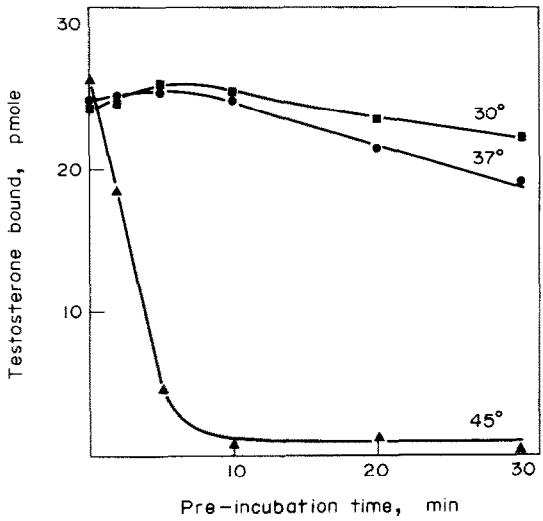

Fig. 2. Effect of pre-incubation temperatures on binding activity. Bacteria induced at 30°C were washed in Tris-HCl, pre-incubated at 30, 37 and 45°C for the periods indicated, and cooled to 4°C prior to assaying for binding activity. The reaction mixture for binding assay contained 0·3 ml of bacteria and 80 pmol of [7-^{3}H]-testosterone ($2·7 \times 10^6$ d.p.m./nmol). Binding was determined after 10 min at 4°C.

Table 3. Induction of binding activity by various steroids

Cultures induced by	[7-^{3}H]-testosterone bound (pmoles)
Testosterone	11·3
Androstenedione	17·1
Androsterone	10·4
5α-Dihydrotestosterone	10·5
5β-Dihydrotestosterone	7·7
Dehydroepiandrosterone	8·7
Progesterone	7·4

Table 4. Binding of C19 and C21 steroids by *P. testosteroni* induced by testosterone

Steroid	Steroid bound (pmoles)
Androstenedione	3·1
Testosterone	2·5
17α-Hydroxyprogesterone	7·6
Progesterone	4·2
Aldosterone	0·2
Cortisol	0·5

steroids, progesterone and 17α-hydroxyprogesterone, which are steroids capable of supporting growth of this organism were bound. On the other hand, aldosterone and cortisol, which are unable to support growth of this organism, were not bound to any significant degree.

Pattern of induction of binding and dehydrogenase activity

The induction of several dehydrogenase activities during adaptive growth of *P. testosteroni* on testosterone has been described by Talalay and his co-workers [7]. These enzymes include 1-ene-dehydrogenase, 4-ene-5α-dehydrogenase, 4-ene-5β-dehydrogenase and 3β(17β)hydroxy steroid dehydrogenase. The sequence of induction of binding and 1-ene-dehydrogenase activities was examined in cultures induced by testosterone at 30°C. 1-ene-Dehydrogenase activity appeared 9 h after induction, reached a maximum at 12 h, and rapidly declined thereafter (Fig. 3). Binding activity also appeared at 9 h, but reached its maximum at about 30 h and persisted much longer. The decline in dehydrogenase and binding activities appeared to be related to depletion of testosterone in the culture medium. Both activities could be re-induced by addition of testosterone after completion of the first induction phase.

Although 1-ene-dehydrogenase activity has been previously reported [6] the conversion of tritium from C7 to ^3H_2O has not been hitherto investigated. When induced cells were incubated in the presence of [1,2,-^{3}H]- or [7-^{3}H]-testosterone at 30°C for 10 min, almost all of the tritium label of the steroid was recovered as tritiated water (Table 5). In the presence of uninduced cells no ^3H_2O was detect-

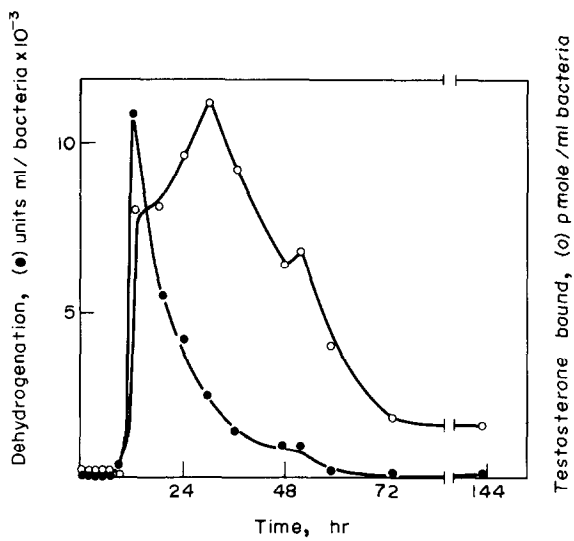

Fig. 3. Induction of binding and dehydrogenation activities. Bacteria were grown at 30°C to a density of $5{\cdot}6 \times 10^9$ cells/ml and induced by the addition of testosterone to a final concentration of 0·5 g/l. At various times after addition of testosterone, cells were assayed for binding and dehydrogenation activities. Binding was assayed on cells washed with 50% ethanol. The reaction mixture for binding activity contained 0·1 ml of bacteria and 2 pmol of [1,2-^{3}H]testosterone (1×10^8 d.p.m./nmol). *E. coli* was used to determine non-specific binding. The reaction mixture for *dehydrogenation* activity contained 36 pmol of [1,2-^{3}H]-testosterone ($5{\cdot}1 \times 10^6$ d.p.m./nmol) and appropriate volumes of bacteria.

Table 5. Fate of radioactive testosterone incubated with induced *P. testosteroni*

Steroid	^3H_2O (% of radioactivity in medium)
[1,2-^{3}H]-Testosterone	95·4
[7-^{3}H]-Testosterone	92·3

able. The formation of ^3H_2O from [7-^{3}H]-testosterone was less rapid and less extensive than that from [1,2-^{3}H]-testosterone (Figs. 4 and 5). Tritium loss from [7-^{3}H]-testosterone was maximal at 30°C with limited conversion to H_2O at 4, 37 and 45°C during 30 min of incubation (Fig. 6). On the other hand, 1-ene-dehydrogenase activity at 37°C was initially equal to that at 30°C.

The ability of induced organisms to degrade [7-^{3}H]-testosterone was examined at various times after induction. The pattern of tritium loss from [7-^{3}H]-testosterone was identical to that of 1-ene-dehydrogenase activity shown in Fig. 3. Since a specific 7-dehydrogenase activity has not been reported for *P. testosteroni*, it is likely that loss of tritium from C7 represents degradation of the steroid molecule.

Inhibition of steroid dehydrogenation and binding activities

When induced cells were washed with 50% ethanol, 1-ene-dehydrogenase activity was inhibited, while binding activity was unaffected or slightly enhanced

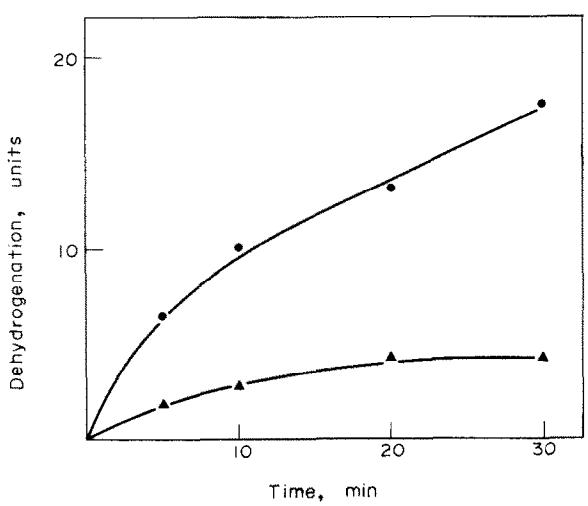

Fig. 4. Dehydrogenation of [1,2-^{3}H]atestosterone and [7-^{3}H]-testosterone at 30°C. The reaction mixture contained in 1 ml: 0·3 ml of bacteria induced for 40 h, 50 mM. Tris-HCl, pH 9·0, and 70 pmol of [1,2-^{3}H]-testosterone ($2·8 \times 10^6$ d.p.m./nmol) (●) or [7-^{3}H]-testosterone ($2·1 \times 10^6$ d.p.m./nmol) (▲). The reaction mixture was incubated at 30°C for periods up to 30 min. The reaction was terminated by the addition of 0·3 ml of dextran-coated charcoal, and the ^3H_2O in the supernatant was determined after removal of the dextran-coated charcoal and bacteria by centrifugation.

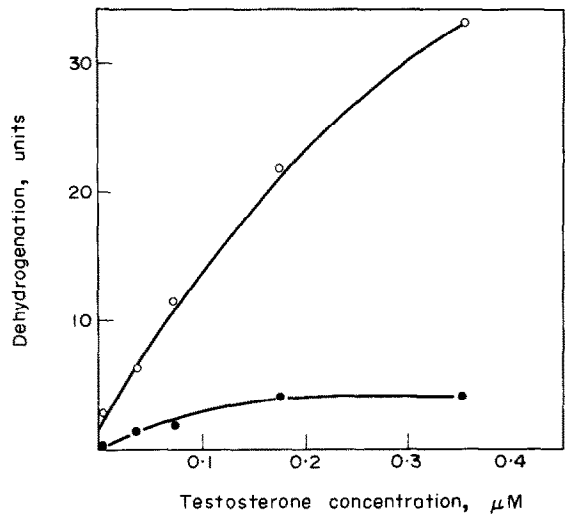

Fig. 5. Effect of substrate concentration on dehydrogenation activity. The experiment was identical to that of Fig. 4, except that increasing concentrations of testosterone were used and the reaction was terminated after 10 min at 30°C. [1,2-^{3}H]-testosterone (○), [7-^{3}H]-testosterone (●).

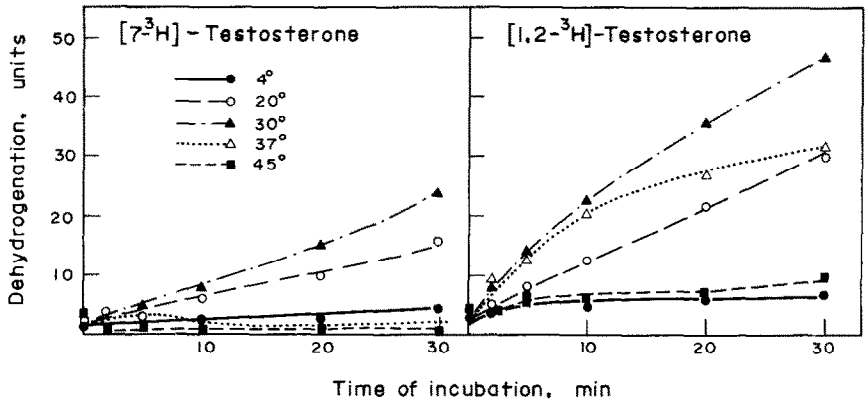

Fig. 6. Effect of temperature on tritium loss from [7-^{3}H]-testosterone and [1,2-^{3}H]-testosterone. The reaction mixture contained in 1 ml: 50 mM Tris, pH 9·0, 85 pmol of [7-^{3}H]-testosterone ($4{\cdot}5 \times 10^6$ d.p.m./nmol) or [1,2-^{3}H]-testosterone ($3{\cdot}3 \times 10^6$ d.p.m./nmol), and 20 μl of Tris-washed bacteria induced for 20 h. The mixture was incubated at the temperatures indicated for periods up to 30 min. All time points designated as 0 min of incubation entailed a 10 min centrifugation at 25°C, except for the 4°C incubation when the centrifugation temperature was also 4°C.

(Table 6). N-ethylmaleimide also inhibited tritium loss from both [1,2-^{3}H]- and [7-^{3}H]-testosterone and produced perhaps a slight increase in binding activity (Table 7).

DISCUSSION

Binding proteins, investigated in a number of bacterial systems, have been implicated in the transfer of solutes across bacterial membranes [8–13]. We have

Table 6. Effect of 50% ethanol on binding and tritium loss from [1,2-^{3}H]-testosterone

Cells washed with	Steroid bound (pmoles)	Steroid dehydrogenated (pmoles)
Tris buffer	3·4	45·9
50% ethanol–water	3·8	0

Table 7. Effect of 5 mM N-ethylmaleimide on binding and dehydrogenation activities of induced *P. testosteroni*

Steroid	Steroid bound (pmoles)	Steroid dehydrogenated (pmoles)
[1,2-^{3}*H*]Testosterone		
without NEM	3·4	45·9
with NEM	5·2	22·4
[7-^{3}H]Testosterone		
without NEM	3·2	2·8
with NEM	3·1	0·6

demonstrated the induction of a steroid-binding activity in *P. testosteroni* and this steroid receptor can be released by exposure of induced cells to osmotic shock [4]. The exact nature of the receptor thus released has not been fully elucidated, although it appears to be a protein with a sedimentation coefficient of approximately 4 S in sucrose density gradient centrifugation. Although indirect evidence suggests that the steroid receptor is a unique protein, the possibility remains that the steroid is bound to one or more of the adaptive enzymes induced during growth of this organism [6]. The pattern of induction of steroid receptor differed appreciably from the induction of overall steroid degradative activity, (as measured by tritium loss from C7) the latter appearing earlier and more transiently than the former. Although we have as yet not examined the various enzyme activities, the 1-ene-dehydrogenase activity appears to be distinct from receptor activity. As with overall degradative activity 1-ene-dehydrogenase activity appears earlier and more transiently than binding activity. The osmotic shock protein does not contain significant 1-ene-dehydrogenase activity (Watanabe, M., unpublished observations) and semi-purified 1-ene-dehydrogenase activity reveals a temperature stability which is different from that of the receptor protein. The receptor protein was unstable at temperatures above 30 and was destroyed at 45°C whereas 1-ene-dehydrogenase activity was greater at 45 than at 30 or 37°C (Watanabe, M., unpublished observations). Finally N-ethylmaleimide inhibited 1-ene-dehydrogenase activity and over all degradative activity without affecting steroid receptor activity. It is possible, however, that other enzymes may be involved in binding steroid. We are currently involved in purification of this steroid receptor in order to investigate this possibility. It should be noted, however, that any associated enzymatic activity need not imply that binding is fortuituous, coincidental, or biologically non-significant. For example, steroid transport may occur through a series of enzymatic transformations as it progresses through the cell

membrane. Such transformations during transport have been reported for other solutes and is termed group translocation[14]. The eventual elucidation and relationship of the steroid receptor to adaptive enzymes will be possible through purification of this protein and through genetic manipulations. Selections of mutants deficient in one or more activity is currently in progress.

Although the role of the binding activity in steroid transport remains to be investigated, the data presented here provide indirect evidence that the binding protein is biologically significant. Binding activity was detectable only under conditions which allowed growth of the organism on steroids. At 37 and 45°C there was poor or no growth of the organism, and no induction of binding activity. The binding activity was reversibly damaged at 37°C and irreversibly damaged at 45°C indicating that the functional integrity of the binding activity was essential for growth on steroids. Furthermore, steroids capable of promoting growth of this organism acted as inducers of binding activity and such steroids were bound whereas steroids unable to promote growth were not bound.

These results suggested that the characteristics and nature of the binding protein determined the growth characteristics of this organism. It will be possible to test this hypothesis by selecting binding protein mutants capable of growth at temperatures over 30°C as well as mutants which might grow on other steroids such as aldosterone and cortisol. A search for such mutants is in progress at the present time.

Binding activity was first detected 9 h after growth on testosterone and the persistence of this activity depended upon the continued presence of testosterone in the medium. Similarly, dehydrogenase activity was dependent upon continued presence of steroid but the rate of disappearance of dehydrogenase activity was more rapid than that of binding activity. The greater binding activity of cultures induced at 25°C as compared to cultures induced at 30°C was no doubt related to greater steroid concentrations present in the medium at 25°C due to less rapid utilization of the steroid at this temperature. Thus, although greater binding activity was detected at 25°C, the optimum growth temperature would appear to be 30°C.

Adaptive enzymes induced during growth of *P. testosteroni* eventually converts the steroid to CO_2 and H_2O[6]. This overall degradative process is very efficient and the amount of steroid converted is much larger than the amount of steroid bound. This efficient degradative activity influences the apparent binding activities by decreasing the amount of radioactive steroid in the reaction mixture. Precautions taken in order to demonstrate binding included conditions which minimized the degradative process and use of high steroid concentrations in the assay. Under these conditions correction was not applied for decrease in amount of radioactive steroid remaining in the incubation mixture. The assay was best performed at 4°C with a short period of incubation. Cultures induced for 30–40 h had relatively little dehydrogenation activity but retained significant binding activity. Finally, dehydrogenation could be inhibited by washing cells with 50% ethanol or by addition of N-ethylmaleimide to reaction mixtures.

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REFERENCES

1. Talalay P., Dobson M. M. and Tapley D. F.: *Nature* **170** (1952) 620.
2. Marcus P. I. and Talalay P.: *J. biol. Chem.* **218** (1956) 661.
3. Talalay P. and Marcus P. I.: *J. biol. Chem.* **218** (1956) 675.
4. Watanabe M., Phillips K. and Chem T.: *J. steroid Biochem.* **4**, 21 (1973) 613.
5. Vaughan B. E. and Boling E. A.: *J. Lab. Clin. Med.*: **57** (1961) 159.
6. Levy H. R. and Talalay P.: *J. biol. Chem.* **234** (1959) 2009.
7. Talalay P.: *Ann. Rev. biochem.*: **34** (1965) 347.
8. Anraku Y.: *J. biol. Chem.* **242** (1967) 793.
9. Anraku Y.: *J. biol. Chem.* **423** (1968) 3128.
10. Kundig W., Kundig F. D., Anderson B. and Roseman S.: *J. biol. Chem.* **241** (1966) 3243.
11. Pardee A. B., Prestidge L. S., Whipple M. B. and Dreyfuss J.: *J. biol. Chem.* **241** (1966) 3962.
12. Piperno J. R. and Oxender D. L.: *J. biol. Chem.* **241** (1966) 5732.
13. Wilson O. H. and Holden J. T.: *J. biol. Chem.* **244** (1969) 2743.
14. Kaback H. R.: *Biochem. biophys. Acta* **265** (1972) 367.