Journal of Steroid Biochemistry, 1973, Vol. 4, pp. 613–621. Pergamon Press. Printed in Great Britain

STEROID–RECEPTOR IN *PSEUDOMONAS TESTOSTERONI* RELEASED BY OSMOTIC SHOCK

MAMORU WATANABE, KEITH PHILLIPS and THOMAS CHEN
Departments of Medicine and Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G3

(*Received* 2 *May* 1973)

SUMMARY

An inducible steroid-binding activity, demonstrated in cultures of ***Pseudomonas testosteroni***, was released into the medium after exposure of bacteria to osmotic shock. The protein nature of the binding activity released into the osmotic shock fluid was indicated by its susceptibility to pronase digestion and heat denaturation and resistance to RNase and DNase. The receptor was saturable at low hormone concentrations and had a high affinity for steroids (K_d $6{\cdot}7 \times 10^{-9}$ M). It formed a complex with binding steroids which had a sedimentation coefficient of approximately 4 S in sucrose density ultracentrifugation studies.

INTRODUCTION

ALTHOUGH the interaction of steroid hormones with intracellular receptors has been investigated in a number of systems, there have been relatively few studies of transport of these compounds across membrane barriers. In mammalian systems, it has been assumed that the passage of steroid hormones across the plasma membrane occurs by simple diffusion [1, 2] whereas transport through the nuclear membrane largely depends upon association with a specific cytoplasmic receptor [3–5].

Studies in bacterial systems have provided considerable information regarding the molecular basis of transport across cell membranes [6–8]. Binding proteins have been isolated which are considered to function as integral components of transport systems [9–14]. In an attempt to delineate a transport system for steroids, we have utilized the bacterium *Pseudomonas testosteroni*. This organism was chosen for these investigations since it is capable of utilizing steroids as a sole carbon source [15] and therefore is likely to have a specific and efficient transport mechanism for such molecules. In this report, we describe the evidence for induction of specific steroid-binding activity, the release of this activity by exposure of induced cells to osmotic shock, and the protein nature of this binding activity.

EXPERIMENTAL PROCEDURES

Materials

P. testosteroni 11996 was obtained from American Type Culture Collection, Rockville, Maryland; unlabeled testosterone from Mann Research Laboratories, New York, New York; RNase A, DNase and pronase from Worthington Biochemical Corporation, Freehold, New Jersey; and Aquasol and [7-^{3}H] testosterone (25 Ci/mmol) from New England Nuclear Corporation, Boston, Massachusetts.

Methods

Growth conditions. Bacteria were grown at 30°C in a medium containing per l: 1 g $NH_4H_2PO_4$, 1 g $(NH_4)_2HPO_4$, 2 g KH_2PO_4, 10 g Difco yeast extract and 10 ml of a solution containing per l: 20 g $MgSO_4 \cdot 7H_2O$, 1 g NaCl, 0·5 g $ZnSO_4 \cdot 7H_2O$, 0·5 g $MnSO_4 \cdot 3H_2O$, 0·05 g $CuSO_4 \cdot 5H_2O$ and 10 ml 0·1 N H_2SO_4.

For induction of binding activity, *P. testosteroni* was grown at 30°C to stationary phase in a gyrotory shaker and testosterone was added to final concentration of 0·5 g/l. Cultures were further incubated at 30°C for 40 h in all studies. Uninduced stationary phase cultures of *P. testosteroni* usually had a λ_{660} of 2·0 with a cell density of $1{\cdot}3 \times 10^{10}$ colony-forming units/ml. After 40 h of growth on testosterone, the λ_{660} was 3·0.

Induced bacteria were harvested by centrifugation at 3,000 *g* for 10 min at 4°C, washed once with 2 volumes of 10 mM Tris-HCl, pH 9·0, and resuspended to the original volume in the same buffer for assay. Uninduced bacteria and *Escherichia coli* cells, used as controls, were resuspended in the same buffer to yield the same λ_{660} as induced cultures of *P. testosteroni*. Cells were stored at 4°C until assayed.

Steroid preparation. A stock solution of radioactive testosterone was prepared in redistilled ethanol to provide a concentration of 100 μCi/ml. For the binding assay, an aqueous solution of labeled testosterone in a concentration of 5 μCi/ml was prepared by evaporating an appropriate volume of stock solution under a stream of nitrogen at 45°C and redissolving the dried steroid in sterile water. Radio-chemical homogeneity of the labeled steroid was checked by paper or thin-layer chromatography.

Unlabeled testosterone was dissolved in either propylene glycol or redistilled ethanol to provide a stock solution with a final concentration of 1 to 10 mg/ml. For the binding assay the testosterone solution was diluted in sterile water to a concentration of 1 μg/ml or less. The small amount of propylene glycol or ethanol transferred to the reaction mixture did not affect binding.

Assay for binding activity of bacterial cells. The reaction mixture for assay of binding activity contained in 1 ml, 0·2–0·3 ml induced or uninduced bacteria, 50 mM Tris-HCl buffer, pH 9·0, and about 10^{-7} M radioactive testosterone of known specific activity. After 10 min of incubation at 4°C, the cells were harvested by centrifugation at 3000*g* for 10 min at 4°C. The bacterial pellet was resuspended in 1 ml of 50 mM Tris-HCl buffer, pH 9·0, and 0·5 ml of the suspension was added to 10 ml of Aquasol. Radioactivity was determined in a liquid scintillation spectrometer. Binding activity was defined as steroid associated with the bacterial cells. Non-specific trapping of radioactivity by the bacterial pellet was determined by using an equal volume of *E. coli* cells as control. This value was subtracted from all binding measurements.

Preparation of osmotic shock protein. Bacterial cells were grown at 30°C to stationary phase and induced by the addition of testosterone to a final concentration of 0·5 g/l. All cultures were induced for 40 h and stored at 4°C until further use.

Cells harvested by centrifugation at 8000 *g* for 10 min at 4°C were washed twice with 0·15 M NaCl, 50 mM Tris-HCl, pH 9·0. Each gram of wet cells was resuspended, with stirring, in 40 ml of 30% (w/v) sucrose, 50 mM Tris-HCl, pH 9·0, and EDTA was added to a final concentration of 5 mM. The mixture was incubated in a rotatory shaker at 25°C for 60 min and the cells were harvested by centrifugation at 8000 *g* for 30 min. The cells were resuspended in a small volume

of 30% sucrose, rapidly dispersed in an original volume of ice-cold buffer (1 mM Tris, pH 9·0, 0·5 mM $MgCl_2$ and 1 mM dithiothreitol), and gently stirred for 30 min at 4°C. Cells were then removed by centrifugation at 25,000 *g* for 30 min. The supernatant thus obtained (referred to as the shock fluid) was concentrated 20 fold by ultrafiltration through a UM-2 Diaflo filter under 80 lb/in^2 nitrogen pressure. Streptomycin sulfate was added to a final concentration of 1% (w/v) and the precipitated DNA removed by centrifugation. The supernatant was dialyzed against 100 volumes of 50 mM Tris, pH 9·0, containing 5 mM EDTA and 1 mM dithiothreitol, for 20 h at 4°C. Protein concentration in the shock fluid was determined by the method of Lowry [16].

Binding assay of osmotic shock protein. Binding of steroids by osmotic shock protein was determined by equilibrium dialysis. Dialysis bags were boiled in 50 mM Tris-HCl, 5 mM EDTA, for 2 h prior to use. Concentrated osmotic shock protein, in a total volume of 1·0 ml, was equilibrated with 15 ml of dialyzing buffer containing 5 mM EDTA, 0·5 mM dithiothreitol, and tritiated steroids in 50 mM Tris-HCl, pH 9·0, at 4°C for 20 h. At the end of dialysis, tritium concentration inside and outside the dialysis bag was determined by adding 0·5 ml of the sample into 10 ml of Aquasol and counting in a liquid scintillation spectrometer. The difference between radioactivity inside and outside the dialysis bag was used to calculate the amount of steroid bound.

Sucrose density gradient centrifugation. Samples of [^{3}H] steroid-protein complex prepared by equilibrium dialysis (usually 0·2 ml) were layered on a linear 5-20% sucrose gradient containing 10 mM Tris, 50 mM NaCl, pH 7·2. The gradients were centrifuged at 4°C in a Beckman Ultracentrifuge Model L2–65B SW 56 rotor at 40,000 rev./min for 15 h. The gradients were then fractionated by piercing the bottom of the tube and collecting 5 drops/vial. Samples were counted in a Nuclear-Chicago Unilux liquid scintillation spectrometer after the addition of 10 ml of Aquasol. Sedimentation coefficients were estimated by using bovine serum albumin (4·6 S) as a marker.

RESULTS

Binding or accumulation of steroids by induced cultures

In order to demonstrate the ability of *P. testosteroni* to either transport or accumulate steroid hormones, initial attempts were directed at demonstrating a higher level of radioactive steroid in induced cultures than in uninduced cultures. Cell-associated radioactivity was measured by harvesting cells by centrifugation, resuspending the bacterial pellet in a small volume of buffer and counting the radioactivity in the resuspended pellet. When cultures induced for 40 h were incubated with radioactive testosterone, a significant proportion of the steroid was found in association with the cells (Fig. 1) and the amount associated with intact bacterial cells was dependent upon the concentration of testosterone added to the reaction mixture (Fig. 2). In contrast, only minimal amounts of testosterone were associated with the bacteria in uninduced cultures. Other bacteria, such as *E. coli*, failed to bind or accumulate steroid.

Radioactive steroid associated with the bacteria could represent steroid bound to the cell surface or steroid accumulated intracellularly. Indirect evidence suggested that the steroid was predominantly bound to the cell surface. Association of radioactive steroid to bacteria occurred after very short periods of incubation and the radioactive steroid could be easily displaced from the cell by repeated

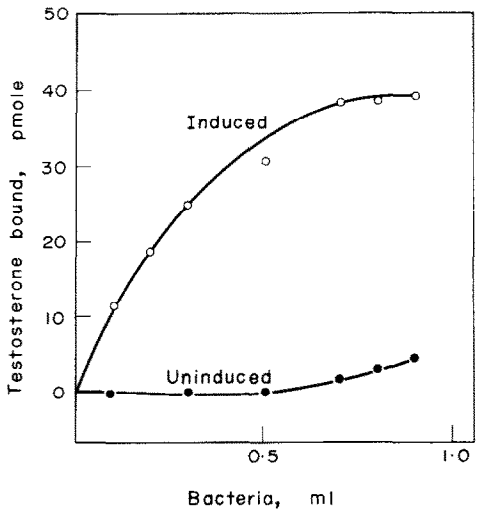

Fig. 1. Binding activity of induced and uninduced *P. testosteroni.* Bacteria grown in the presence of testosterone for 40 h (○) or in its absence (●) were harvested by centrifugation, washed with Tris-HCl and resuspended in 10 mM Tris-HCl, pH 9·0, at 4°C. The reaction mixture for assay of binding activity contained 80 pmoles of [7-^{3}H]testosterone ($2{\cdot}7 \times 10^6$ d.p.m./nmole) and volumes of bacteria as indicated. After 10 min at 4°C the bacterial cells were harvested by centrifugation and the radioactivity associated with the bacterial pellet was determined. The amount of steroid trapped non-specifically by the bacterial pellet was determined using an equal volume of *E. coli* cells and the corresponding value was subtracted to yield the data shown.

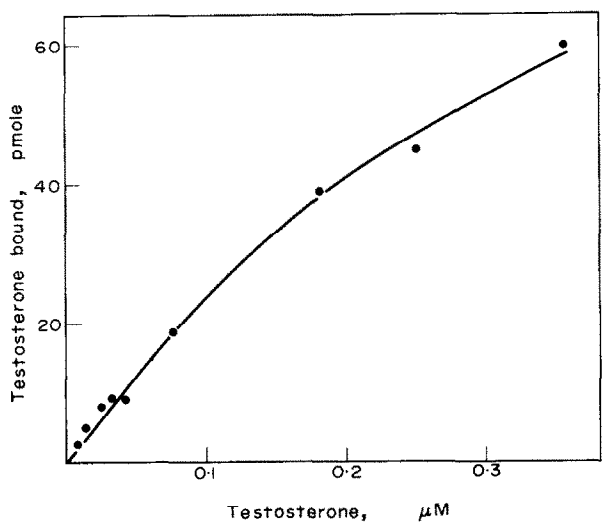

Fig. 2. Effect of testosterone concentration on amount of steroid bound by induced cultures. The assay mixture contained 0·2 ml of Tris-washed bacteria, and various concentrations of [7-^{3}H]testosterone. Binding was determined by centrifugation as outlined in Fig. 1.

washing. Steroids associated with bacteria, under conditions of our assay, have therefore been referred to as "bound".

Release of binding activity by osmotic shock

In order to characterize the steroid-binding activity of bacterial cells, attempts were made to isolate it from induced cultures. Exposure of induced *P. testosteroni* to osmotic shock resulted in the release of binding activity into the shock fluid (Table 1). The amount of radioactive testosterone bound was related to the amount of osmotic shock protein (Fig. 3). Shock fluid prepared from uninduced cultures contained approximately 10% of the binding activity of shock fluid from induced cells, indicating a low level of constitutive activity (Table 1).

Table1. Binding of [7-^{3}H] testosterone by osmotic shock fluid

Bacteria	Steroid concentration (nM)	Steroid (bound/free)	Steroid bound (pmol/mg protein)
Uninduced	13	0·14	5·4
	26	0·21	15·4
Induced	13	1·46	46·5
	26	2·61	104·1

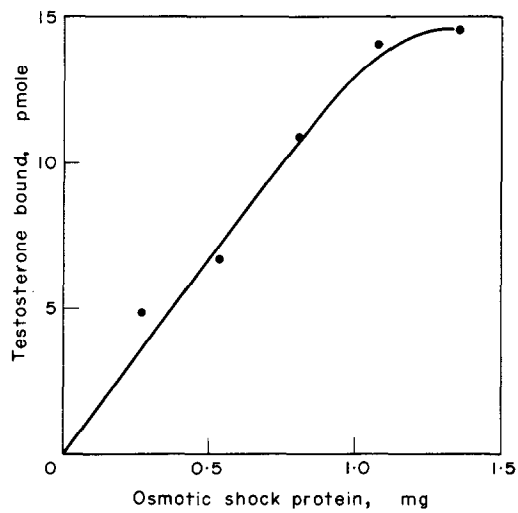

Fig. 3. Binding of testosterone by osmotic shock protein. Osmotic shock protein was prepared from cultures induced for 40 h and binding was assayed by equilibrium dialysis as described in the text. The dialysis medium contained 5·3 nM [7-^{3}H]testosterone ($2·5 \times 10^7$ d.p.m./nmol) in addition to materials described in the text. Binding was assayed using various amounts of osmotic shock protein.

Mild conditions were employed for shocking which did not destroy the viability of the organisms. The colony-forming ability of the organism was unaffected by shocking, and the growth curve in fresh medium was identical for shocked and unshocked cells.

The effect of testosterone concentration on steroid-binding activity of osmotic

shock fluid was examined. Binding activity of osmotic shock fluid, assayed by equilibrium dialysis, was dependent upon the concentration of testosterone in the dialysis medium (Fig. 4). The Scatchard plot[17], derived from the data shown in Fig. 4, produced a straight line (Fig. 5). The equilibrium constant for the dissociation reaction (using osmotic shock fluid) was estimated to be about $6{\cdot}7 \times 10^{-9}$ M.

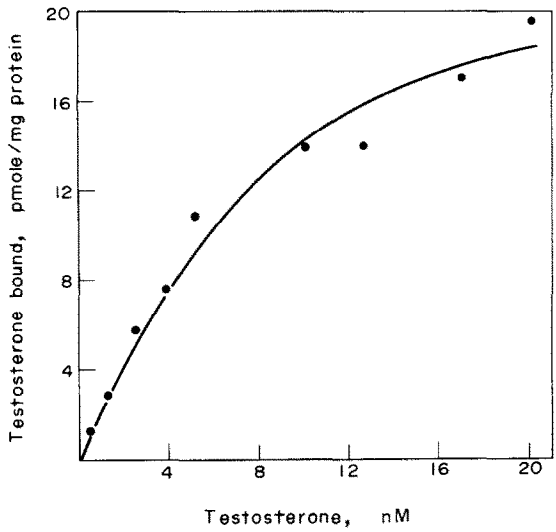

Fig. 4. Effect of testosterone concentration on binding activity of osmotic shock protein. Binding activity was tested at various testosterone concentrations, using 1·35 mg of osmotic shock protein.

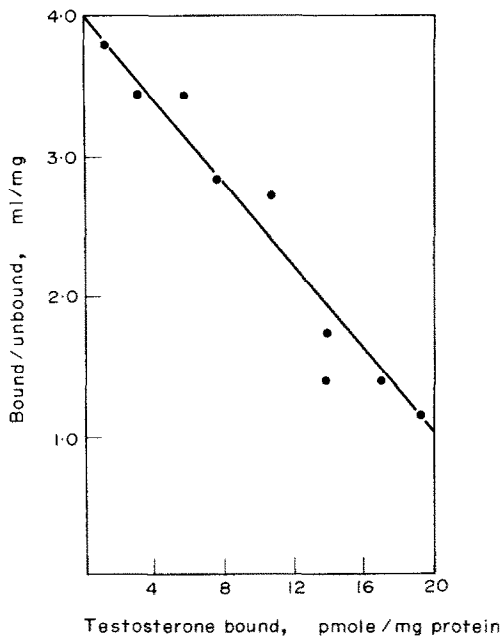

Fig. 5. Scatchard plot of testosterone binding by osmotic shock protein. The plot was derived from data shown in Fig. 4.

The possibility of a second set of sites with a lower affinity resulting in a bound/unbound ratio of less than 1 has not yet been excluded.

Characterization of binding activity in osmotic shock fluid

The nature of the binding activity released by osmotic shock was partially characterized. Binding activity of osmotic shock fluid was rapidly destroyed by incubation at 45°C (Fig. 6). Binding activity was destroyed by treatment with pronase, whereas RNase and DNase digestion had no effect (Table 2). Binding was not inhibited, and perhaps was somewhat enhanced by N-ethylmaleimide. Binding activity was not affected by pH between the range of 6 to 10 (Table 3).

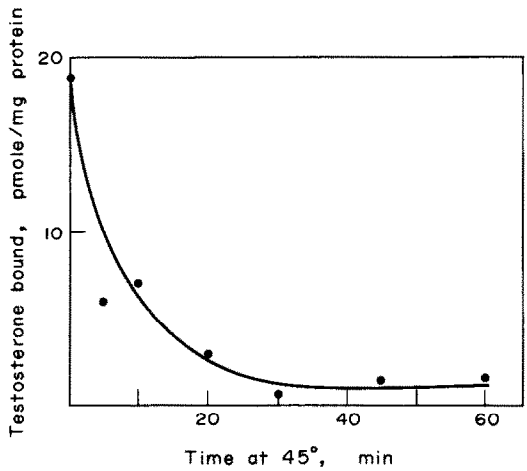

Fig. 6. Effect of pre-incubation at 45°C on binding activity of osmotic shock protein. Osmotic shock protein (0·4 mg/assay) was incubated at 45°C for varying periods prior to assaying for binding activity. Dialysis medium contained 7·4 nM [7-^{3}H]testosterone ($2{\cdot}1 \times 10^7$ d.p.m./nmol).

Table 2. Effect of pronase, RNase, DNase and N-ethylmaleimide on binding by osmotic shock protein

Treatment		Testosterone bound (% of control)
Pronase	100 μg/ml, Δ37°C 60 min	2·0
RNase	100 μg/ml, Δ37°C 60 min	108·5
DNase	100 μg/ml, Δ37°C 60 min	100·5
N-ethylmaleimide	10 mM	134·9

Note 1. During pre-treatment with RNase and DNase $MgCl_2$ was added to a concentration of 10 mM.

Note 2. During pre-treatment with pronase, 0·1 M EDTA was added.

Note 3. When N-ethylmaleimide was added, dithiothreitol was omitted from the reaction mixture.

Sucrose density gradient analysis of shock protein

When labeled testosterone was added to the dialysis medium and the dialysate containing shock protein analyzed by sucrose density gradient centrifugation, a single peak of hormone-protein complex was obtained (Fig. 7). There was minor

Table 3. Effect of pH on binding

pH	Testosterone (bound/unbound)
6·0	2·66
7·0	2·41
8·0	2·36
9·0	2·55
10·0	2·21

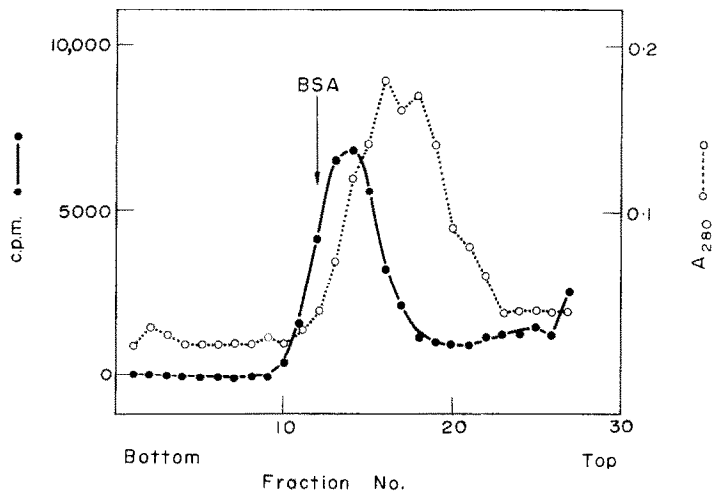

Fig. 7. Centrifugation of [^{3}H]testosterone-labeled osmotic shock protein. Osmotic shock protein prepared from cultures induced for 40 h was dialyzed in the presence of [7-^{3}H]testosterone for 20 h at 4°C. The steroid-protein complex was analyzed on sucrose gradient centrifugation. Bovine serum albumin (↓) was added as a marker in a separate tube. Protein concentration was determined by absorbance at 280 nm (A_{280}) in a spectrophotometer.

variation in its sedimentation constant with salt concentration which was not significant. In 0·01 M KCl the bound hormone had a coefficient of 4·3, whereas in 0·4 M KCl the S value was 3·9.

DISCUSSION

Binding proteins for various molecules have been isolated from several bacteria [9–14]. These proteins are located in the periplasmic space and are considered to function as integral components of transport systems. Evidence for their direct role in transport has included loss of transport activity and concomitant recovery of binding activity in shock fluid after osmotic shock treatment, restoration of transport activity by addition of purified binding proteins to shocked bacteria, similar kinetic constants for transport and binding activity, and coordinate regulation of transport activity and ability to synthesize binding protein [7]. If these binding proteins do not function as "carriers" transporting solutes from the outer to the inner membrane, they may function as "pre-transport" proteins accumulating and concentrating the solutes at the cell surface, transferring the solutes to a specific transport system.

P. testosteroni is an organism capable of utilizing steroids as a sole carbon source[15]. During the course of growth on testosterone, it is expected that the organism will accumulate and transport steroids into the intracellular compartment. Our initial studies were aimed at demonstrating the presence of steroid-binding activity in induced cultures of *P. testosteroni.* This steroid-binding activity, which could be released into the medium by osmotic shock treatment demonstrated characteristics of a protein molecule. Binding activity was destroyed at incubation temperatures at 45°C. Binding activity of osmotic shock protein was destroyed by pronase, indicating a requirement for an intact protein structure. However, thiol groups were not essential for binding.

The osmotic shock protein demonstrated characteristics of a specific steroid receptor. The receptor was saturable at low hormone concentrations and had a high affinity for steroids. (K_d $6{\cdot}7 \times 10^{-9}$ M). The release of this steroid-binding protein by osmotic shock indicated the periplasmic location of the binding activity. The role of this binding protein in the translocation process of steroids across cell membrane remains to be elucidated.

ACKNOWLEDGEMENT

This investigation was supported by grant MA 4425 from the Medical Research Council of Canada, Ottawa, Canada. Dr. Watanabe is an Associate, Medical Research Council of Canada, Ottawa, Canada.

The expert technical assistance of Miss Luisa Po is gratefully acknowledged.

REFERENCES

1. Giorgi E. P., Stewart J. C., Grant J. K. and Scott R.: *Biochem. J.* **123** (1971) 41.
2. Munck A.: *Persp. Biol. Med.* **14** (1971) 265.
3. Jensen E. V., Mohla S., Gorell T., Tanaka S. and De Sombre E. R.: *J. steroid Biochem.* **3** (1972) 445.
4. O'Malley B. W., Toft D. O. and Sherman M. R.: *J. biol. Chem.* **246** (1971) 1117.
5. Shyamala G. and Gorski J.: *J. biol. Chem.* **244** (1969) 1097.
6. Kaback H. R.: *Ann. Rev. Biochem.* **39** (1970) 561.
7. Oxender D. L.: *Ann. Rev. Biochem.* **41** (1972) 777.
8. Pardee A. B.: *Science* **162** (1968) 632.
9. Anraku Y.: *J. biol. Chem.* **242** (1967) 793.
10. Anraku Y.: *J. biol. Chem.* **423** (1968) 3128.
11. Kundig W., Kundig F. D., Anderson B. and Roseman S.: *J. biol. Chem.* **241** (1966) 3243.
12. Pardee A. B., Prestidge L. S., Whipple M. B. and Dreyfuss J.: *J. biol. Chem.* **241** (1966) 3962.
13. Piperno J. R. and Oxender D. L.: *J. biol. Chem.* **241** (1966) 5732.
14. Wilson O. H. and Holden J. T.: *J. biol. Chem.* **244** (1969) 2743.
15. Talalay P., Dobson M. M. and Tapley D. F.: *Nature* **160** (1952) 620.
16. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: *J. biol. Chem.* **193** (1951) 265.