Vol. 109, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1982 Pages 518-526

CHEMICAL MODIFICATION OF OPIATE RECEPTORS WITH ETHOXYFORMIC ANHYDRIDE AND PHOTO-OXIDATION: EVIDENCE FOR ESSENTIAL HISTIDYL RESIDUES

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Received October 5, 1982

Rat-brain membrane preparations containing opiate receptors were ethoxyformylated (EFA) in order to identify the presence of histidine residues at the binding site. A complete loss of binding activity resulted within a few minutes. The inhibitory effect of EFA was reversed by hydroxylamine incubation. The activity of the receptors was found to decrease in a dose-response manner after photo-oxidation with Rose Bengal. When the receptors were pre-incubated with β-endorphin or methionine enkephalin, they were found to be protected from EFA inhibition. These findings suggest that histidine residues may be involved in the binding of opiate receptors to their ligands.

In order to understand the structure and function of a receptor, binding studies using radioactive ligands are needed. Various studies have identified and characterized the opiate receptor by using radioactive ligands of high specific activity (1-3). The use of enzymes and site-specific reagents as investigative probes for understanding the chemical environment of the opiate receptor has proved promising. For example, there have been various reports showing how opiate receptor binding is modified by enzymes, and interesting findings have revealed the nature of the receptor and characteristics of the ligand binding. The stereospecific binding of opiate receptors is reduced when they are treated with enzymes such as pronase, trypsin, and chymotrypsin (3-5). There is a difference in the mode of their inactivation: e.g., trypsin destroys receptor sites, while chymotrypsin reduces the affinity of the receptor and the ligand (4). Opiate receptor binding may be influenced by phospholipases (controversial, 6-10). That receptor binding is also affected by ionic detergents suggests the involvement of phospholipid components in the receptor (11-13, 4).

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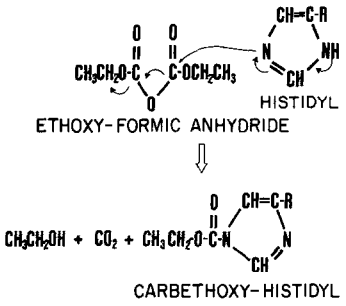

Fig. 1 Chemical Reaction Between a Histidine Residue and Ethoxyformic Anhydride (EFA).

Furthermore, opiate receptor binding has been found to be decreased by protein-modifying agents N-ethyl-maleimide and iodoacetamide. These results indicate the importance of sulfhydryl (SH) groups in opiate binding (3, 7). However, protein-modifying reagents are less effective when the SH group of the receptor site is protected by preincubation with excessive opiates, either agonists or antagonists (14, 15), or when sodium chloride is present (16, 17). Ahmed and Byrne (18) have used a site-specific reagent, 4,4' dithiopyridine, and found inhibition of opiate receptor binding.

In order to understand the mode of action of opiates (including endorphins) as comprehensively as possible, it is vital to have a pure receptor preparation. Success in achieving this has been rather limited, because of the lack of a suitable technique that would solubilize the receptor while maintaining its stereospecific binding properties (19-28). The immediate goal is the development of a method of labelling the binding sites with a reversible reagent which would bind covalently but selectively. Ethoxyformic anhydride (EFA) is a reagent ideal for chemical modification of proteins with a high degree of specificity, and it does not require extreme conditions for modification because it reacts at neutral or near neutral pH in dilute buffers to modify only histidine residues (Fig. 1. 29-31). EFA has been used extensively to study the function of histidine residue in ribosomal proteins (32,33) and also in bovine prolactin (34).

The other approach to identifying histidine residues is by photo-oxidation of the residues in the presence of an anionic dye, Rose Bengal. The availability of an opiate-receptor binding assay method greatly facilitates the measurement of the receptor's activity parallel to its chemical modification (35). In view of these considerations, it was decided to investigate the presence of essential histidine residues in the opiate receptor. Part of this investigation has already been presented elsewhere (36).

MATERIALS AND METHODS

Synaptosomes from the brains of male Wistar rats killed by cervical dislocation were prepared as follows: the brain without the cerebellum was homogenized in sucrose 0.32 M (∿20 ml/g wet tissue) and the suspension was centrifuged at 120 xg for 10 minutes, after which the supernatant was carefully decanted into a beaker and the cell debris discarded. The suspension was incubated for 45 minutes at 37° C and centrifuged for 30 minutes at 50,000 xg; the pellet was resuspended in sucrose and centrifuged again at 12,000 xg for an additional 10 minutes. The complete procedure was repeated three times to dispose of unwanted materials. The resultant pellet was resuspended in sucrose (∿5.3 ml/g wet tissue) and divided into aliquots, which could be thawed, recentrifuged at 12,000 xg, and resuspended in Tris. HCl Buffer (40 mM, pH 7.4) to an appropriate concentration (∿30 ml/1.5 g wet tissue).

Synaptosomal suspensions (200 µl) were placed in a series of assay tubes, and ^{3}H-etorphine (specific activity 38 Ci/m mol) diluted to a concentration of approximately 1nM in 100 µl was added. The assay mixtures were incubated for 45 minutes at room temperature and then rapidly filtered on Whatman Glass Microfibre filters (GF/B) to trap radioactively bound synaptosomes. The filters were washed with cold Tris. HCl buffer and placed in 8 ml of Aquasol liquid scintillation cocktail (New England Nuclear) for several hours. The radioactivity was counted by Beckman LS7500 Liquid Scintillation spectrometry. Specific binding of ^{3}H-etorphine to the receptor was defined as the difference between the amount bound in the presence and in the absence of an excess of unlabelled opiate (e.g., methionine enkephalin) (35).

The specific binding of treated synaptosomes was compared to that of untreated synaptosomes. The amount of protein from synaptosomes was determined spectrophotometrically by the Bio-Rad assay technique (Bio-Rad Laboratories), with bovine serum albumin used as the standard.

Inactivation of receptor binding by ethoxyformic anhydride (EFA): The procedure for the EFA reaction with brain synaptosomes was essentially that described as follows (32): i.e., synaptosomes which had been washed twice in phosphate buffer (10 mM, pH 6.0) were treated for 30 minutes at 0° C with EFA (950 µmol/ml) in methanol. To terminate the reaction, histidine (10 mM) was added, and the mixtures were washed several times in Tris. HCl buffer (50 mM, pH 7.4) and assayed for opiate receptor activity. The gradual inactivation of the receptor under EFA treatment at different concentrations was noted at defined time intervals. As a control, the procedures were applied to synaptosomes not treated with EFA.

Reversibility of EFA-inhibited reaction: Reversibility upon treatment with hydroxylamine has been reported by Melchior and Fahrney (31). In our studies the reactivation of the ethoxyformylated synaptosomes with hydroxylamine was carried out as follows (32): ethoxyformylated synaptosomes were treated for 10 to 20 minutes with hydroxylamine (0.25 M, pH 7.4) at room temperature; then the mixture was centrifuged and the pellets resuspended in Tris. HCl buffer (50 mM, pH 7.4), dialysed for a few hours using the same buffer after several changes, and finally assayed for opiate receptor activity. Controls (without ethoxyformylation) were also treated with hydroxylamine.

Inactivation of pre-treated synaptosomes: In order to study whether the opiate agonist protects synaptosomes against ethoxyformylation, the following experiments were carried out: different concentrations of Met-enkephalin and β-endorphin (in Tris. HCl buffer 0.05 mM, pH 7.4) were

pre-incubated with brain synaptosomes for 15 minutes at room temperature, after which they were cooled to 0° C and treated with EFA (0.5 mM) for 30 minutes. Histidine 10 mM was added to terminate the reaction, and the mixture was washed numerous times with Tris. HCl buffer (50 mM, pH 7.6). Finally, the mixture was assayed for opiate receptor activity. Corresponding controls were not treated with Met-enkephalin or β-endorphin.

Inactivation of receptor binding by photo-oxidation with Rose Bengal: Rose Bengal, an anionic dye, causes a rapid loss of activity of ribosomal proteins under very mild conditions and shows a high degree of specificity for histidine (37). Photo-oxidation of the synaptosomes was carried out using a reaction mixture containing synaptosomes equivalent to 25 mg brain tissue in 1 ml Tris. HCl buffer (50 mM, pH 7.4) and varying concentrations of Rose Bengal (2, 4, and 6 mM). The reaction vials were wrapped in aluminum foil and placed in an ice bath, and the mixture shaken gently for a few seconds to dissolve the maximum amount of air. It was irradiated with a 150 W Westinghouse reflector spotlight held 30 cm directly above for various time intervals (0-40 minutes). Controls were set up for two conditions: absence of dye and absence of light. Samples were washed repeatedly and assayed using ^{3}H-etorphine (38 Ci/m mol was diluted to give concentration of 1nM).

RESULTS AND DISCUSSION

Receptor binding was found to be inactivated by EFA. The loss of specific binding observed was 47.4% at less than 0.1 mM, 50-60% at 0.25 mM, and 95% at 1mM EFA concentrations (Fig. 2). The inactivation process was extremely fast: at 0.5 mM EFA complete inactivation occurred in about one minute. The process took longer at lower concentrations of EFA (Fig. 3)

Reversibility of the EFA-inhibited reaction was observed upon treatment with hydroxylamine. A loss of 63.3% of specific binding activity was noted after treatment with EFA, and a regeneration of 66.2% of activity after treatment with hydroxylamine (Table 1).

Pre-treatment of the synaptosomes with either met-enkephalin or β-endorphin was found to provide significant protection from ethoxyformylation (Fig. 4). This protection effect suggests that EFA is acting at a site very close to the opiate receptor binding site.

Exposure of the synaptosomes to Rose Bengal and to light resulted in loss of specific binding activity (Fig. 5). The higher the concentration of Rose Bengal in the reaction mixture, the faster and greater the loss of activity. In neither control condition (absence of dye, absence of light) was any significant loss of activity observed.

In the above experiments, maximum inactivation of opiate receptors by EFA at pH 6.0 was observed within five minutes, a speed which suggests the accessibility of histidine residues towards inactivation by EFA.

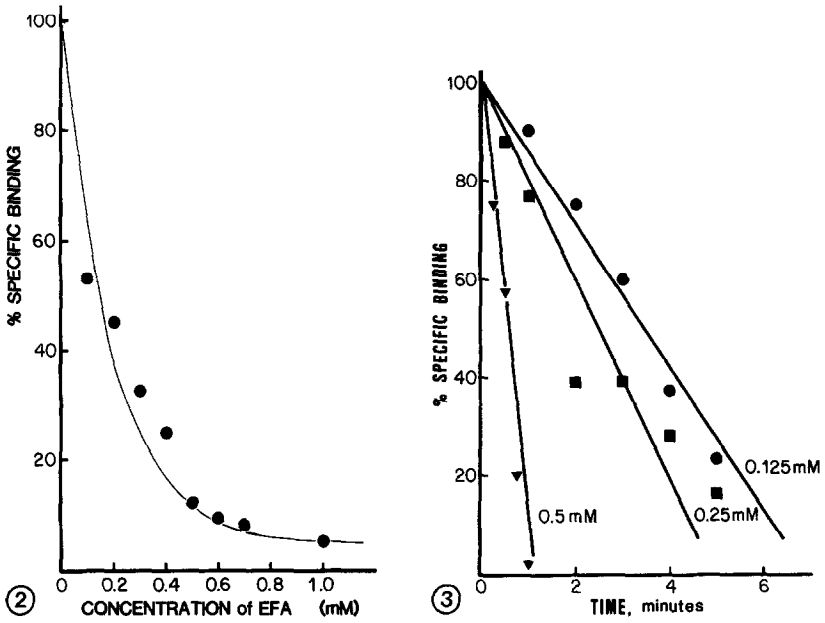

Fig. 2 Inactivation of Receptor Binding by Ethoxyformic Anhydride (Effect of Concentration).
Rat brain synaptosomes were treated with various concentrations of EFA for 30 min. at 0°C. Loss of specific binding ranged between 47.4% and 95%.

Fig. 3 Kinetics of Opiate Receptor Inactivation by Ethoxyformic Anhydride.
At defined intervals the remaining ^{3}H-etorphine binding activity was measured. Rate of inactivation was found to vary directly with EFA concentration.

It has been demonstrated that when proteins are treated with EFA at acidic pH, histidine residues specifically are modified. Muhlrand et al. (37) demonstrated that (besides histidine) arginine, lysine, tyrosine, and sulfhydryl groups can also be EFA-ethoxyformylated at higher pH. However, the reagent is specific for histidine at pH 6.0 (38), and sulfhydryl groups are not modified (39).

Melchior and Fahrney (31) and Wells (40) discovered that amino groups of proteins, such as lysine, may react with EFA, even at low pH (4.6). Reaction with amino groups is not reversible by hydroxylamine, but hydroxylamine can restore the binding activity of the ethoxyformylated histidines. As much as 66.2% binding activity was restored after hydroxylamine incubation of the ethoxyformylated receptors. Thus inactivation of opiate receptor binding activity by EFA and its reversal by hydroxylamine suggests strongly that histidine residues are modified and that there may be one or more residues involved.

Protection of opiate receptors by met-enkephalin and ß-endorphin against inactivation by EFA is further evidence that histidine residues located in the active core of the receptor are essential for binding

TABLE 1

Reversal of EFA Inactivation
of Opiate Receptors by Hydroxylamine

Sample	Specific Binding cpm/mg protein	% Specific Binding	% Loss of Specific Binding
Untreated synaptosomes	6265 ± 284	100	0
Synaptosomes + Hydroxylamine	5355 ± 116	86.2	13.8
Synaptosomes + EFA (30 mins)	2286 ± 39	36.7	63.3
Synaptosomes + EFA + Hydroxylamine (30 mins)	4124 ± 292	66.2	33.8

Synaptosomes obtained as described in Materials and Methods were ethoxyformylated, treated with hydroxylamine (0.25M), and dialysed for a few hours, then assayed for opiate receptor activity. Controls without ethoxyformylation were also treated with hydroxylamine. Each value represents the mean (±S.E.M.) from at least three independent experiments.

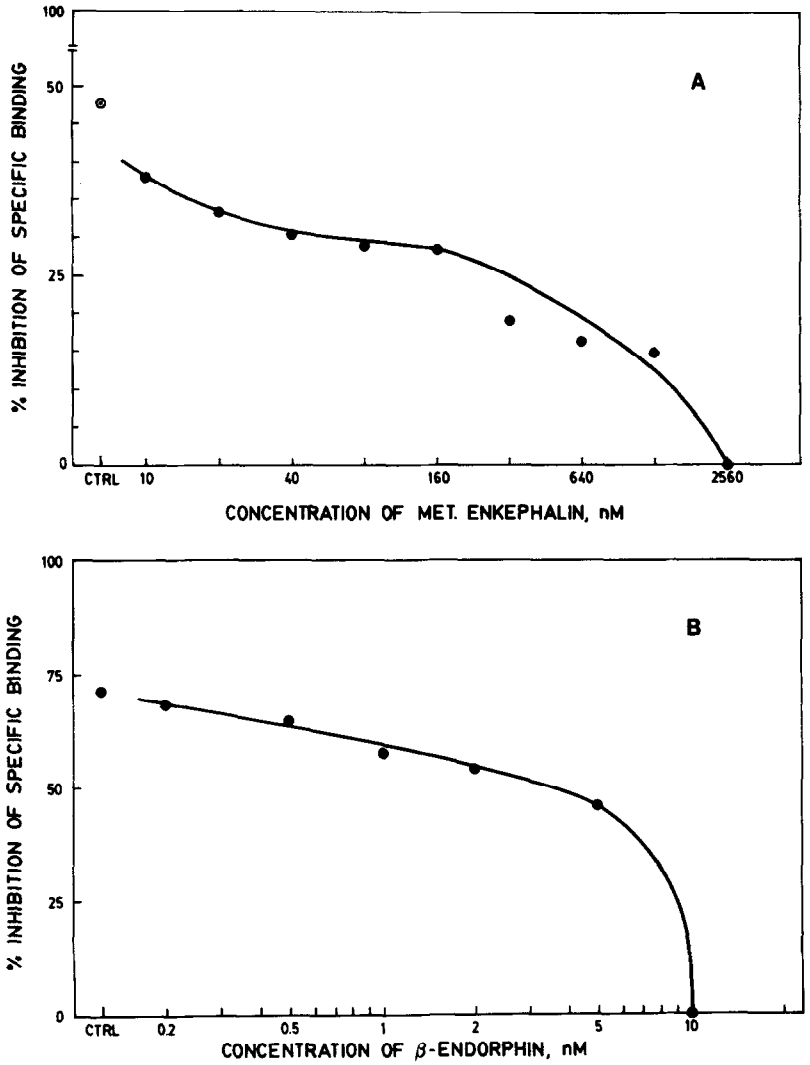

Fig. 4 Protection From EFA Inactivation by Pre-Treatment with (A) Met-Enkephalin and (B) β-Endorphin.

Synaptosomes were pre-incubated with either met-enkephalin or β-endorphin, then treated with EFA. The receptor's ability to bind with ^{3}H-etorphine was then measured. The pre-treatment was found to provide significant protection from ethoxyformylation. Corresponding controls (CTRL) were not treated with met-enkephalin or β endorphin.

activity. It is also possible, however, that they protect the receptor binding by changes which are induced by allosteric interactions.

Photo-oxidation was used as another probe to examine the possible involvement of histidine residues in opiate receptor binding. Ray (41) reported that photo-oxidation destroyed cysteine, methionine, tyrosine, and tryptophan, as well as histidine residues in proteins. The anionic dye Rose Bengal has been shown to destroy histidine residues (42). In this investigation, inactivation of opiate receptors at 2 mM - 6 mM con-

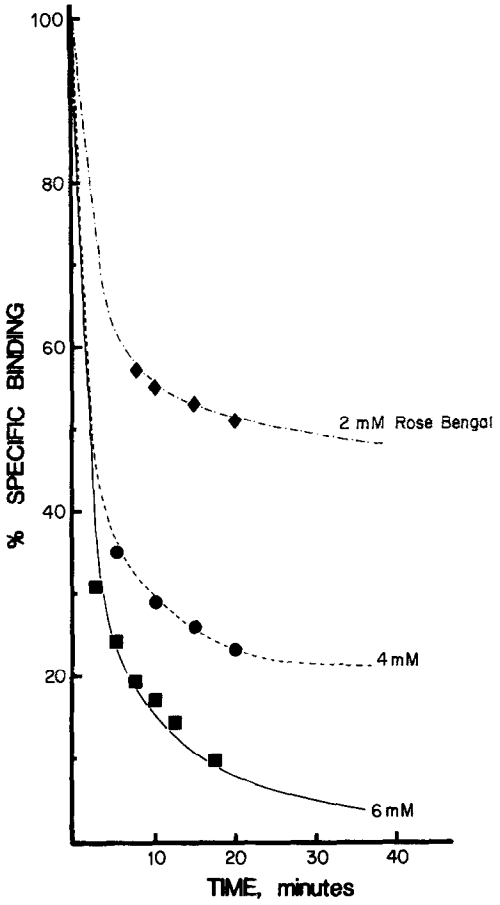

Fig. 5 Inactivation of Receptor Binding by Photo-oxidation with Rose Bengal.

Synaptosomes were mixed with Rose Bengal at various concentrations and irradiated for various time intervals. The higher the concentration of Rose Bengal in the mixture, the faster and greater the loss of specific binding activity. Control incubations were carried out (a) in absence of dye and (b) in absence of light.

centrations of Rose Bengal, in a dose-response manner, again suggests that histidines are involved in opiate receptor binding.

In conclusion, we have put forward evidence that histidine residues are essential for the binding of the opiate receptor to its ligand. Affinity columns of EFA could be made and the opiate receptor activity could be regenerated by treatment with the hydroxylamine. This may be a promising technique for purification of the receptor.

ACKNOWLEDGMENTS

Grateful acknowledgement is given to Dean R.M. Baxter for his encouragement throughout this work, and to Messrs. J. Go and N. Zahid for their expert technical assistance.

REFERENCES

1. Goldstein, A., Lowney, L.I. and Pal, B.K. (1971) Proc . Natl. Acad. Sci. U.S.A. 68, 1742-1747.
2. Pert, C.B. and Snyder, S.H. (1973) Sci. 179, 1011-1014.
3. Simon, E.J., Hiller, J.M. and Edelman, I. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 1947-1949.
4. Pasternak, G.W. and Snyder, S.H. (1974) Molec. Pharmacol. 10, 183-193.
5. Pasternak, G.W. and Snyder, S.H. (1975) Molec. Pharmacol. 11, 478-484.
6. Wilson, H.A., Pasternak, G.W. and Synder, S.H. (1975) Nature 253, 448-450.
7. Lin, H.K. and Simon, E.J. (1978) Nature 26, 383-384.
8. Abood, L.G., Salem, N., MacNeil, M. and Butler, M. (1978) Biochem. Biophys. Acta 530, 35-46.
9. Lin, H.K. and Simon, E.J. (1978) Nature 271, 383-384.
10. Ruegg, U.T., Cuenod, S., Fulpius, B.W. and Simon, E.J. (1982) Biochem. Biophys. Acta 685, 241-248.
11. Terenius, I.(1973) Acta Pharmacol. 33, 317-320.
12. Abood, L.G. and Takeda, F. (1976) Europ. J. Pharmacol. 39, 71-77.
13. Abood, L.G., Salem, N., MacNeil, M., Bloom, L. and Abood, M.E. (1977) Biochem. Biophys. Acta 468, 51-62.
14. Pasternak, G.W., Wilson, H.A. and Snyder, S.H. (1975) Molec. Pharmacol. 11, 340-351.
15. Simon, E.J. and Groth, J. (1975) Proc. Natl. Acad. Sci.U.S.A. 72,2404-2407.
16. Simon, E.J., Hiller, J.M., Groth, J. and Edelman, J. (1975) J. Pharmacol. Exp. Ther. 192, 531-537.
17. Hiller, J.M. and Simon, E.J. (1976) In: Tissue responses to addictive drugs. D.H. Ford and D.H. Clouet (eds.), Spectrum Publications, Inc., New York, pp. 335-353.
18. Ahmed, M.S. and Byrne, W.L. (1979) In: Endogenous and exogenous opiate agonists and antagonists. E. Leongway (ed.), Pergamon Press, N.Y., pp.51-54.
19. Simon, E.J, Hiller, J.M. and Edelman, I. (1975) Science 190, 389-390.
20. Zukin, R.S. and Kream, R.M. (1979) Proc. Natl. Acad. Sci.U.S.A.76,1593-1597.
21. Ruegg, U.T., Cuenod, S., Hiller, J.M., Gioannini, T., Howells, R.D. and Simon, E.J. (1981) Proc. Natl. Acad. Sci.U.S.A. 78, 4635-4638.
22. Cho, T.M., Yamato, C., Cho, J.S. and Loh, H.H. (1981) Life Sci. 28, 2651-7.
23. Bidlack, J.M., Abood, L.G., Osei-Gyimah, P. and Archer, S. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 636-639.
24. Simonds, W.F., Koski, G., Streaty, R.A., Hjelmeland, L.M. and Klee, W.A. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 4623-4627.
25. Ruegg, U.T., Hiller, J.M. and Simon, E.J. (1980) Eur. J. Pharmacol. 64,367-8.
26. Law, P.Y., Herz, A. and Loh, H.H.(1979) J. Neurorhem.33, 1177-1187.
27. Simon, E.J. (1979) Prog. Clin. Biol. Res. 27, 51-62.
28. Bidlack, J.M. and Abood, L.G. (1980) Life Sciences 27, 331-340.
29. Rogers, T.B., Gold, R.A. and Feeney, R.E. (1977) Biochemistry 16, 2299-2305.
30. Chang, G.G. and Hsu, R.Y. (1977) Biochem. Biophys. Acta 483, 228-235.
31. Melchior, W.B. Jr. and Fahrney, D. (1970) Biochemistry 9, 251-258.
32. Baxter, R.M. and Zahid, N. (1978) Eur. J. Biochem. 91, 49-56.
33. Favorova, O., Madoyan, I.A. and Kisseley, L.L. (1978) Eur. J. Biochem 86,193-202
34. Anderson, T.T. and Ebner, E.E. (1979) J. Biol. Chem. 254, 10995-10999.
35. Pert, C.B. and Snyder, S.H. (1973) Proc. Natl. Acad. Sci.U.S.A.70,2243-2247.
36. Roy, B.P., Baxter, R.M., Zahid, N.U.D., Ng, A.Y.H. (1981) CFBS No-111.
37. Muhlrand, A., Hegyi, G., and Toth, G. (1967) Biochem. Biophys. Acta .2, 19-29.
38. Ovadi, J., Libor, S. and Elodi, P. (1967) Biochem. Biophys. Acta 2, 455-458.
39. Pradel, L.A. and Kassab, R. (1968) Biochem. Biophys. Acta 167, 317-325.
40. Wells, M.A. (1973) Biochem. 12, 1086-1093.
41. Ray, W.J. Jr. (1967) Methods Enzymol. 11, 490-497.
42. Westhead, E.W. (1965) Biochem. 4, 2139-2144.