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EFFECTS OF FLUORINE ANALOGS OF NOREPINEPHRINE ON STIMULATION OF CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE AND BINDING TO β-ADRENERGIC RECEPTORS IN INTACT PINEALOCYTES

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Abstract—Alterations in β-adrenergic agonist activity resulting from fluorine substitution on the 2-, 5- or 6-position of the aromatic ring of norepinephrine were investigated, using dispersed neonatal rat pinealocytes. Increases in cyclic AMP in these cells are known to be governed entirely by β-adrenergically coupled adenylate cyclase. Norepinephrine (NE), 2-fluoronorepinephrine (2F-NE), 5-fluoronorepinephrine (5F-NE), and 6-fluoronorepinephrine (6F-NE) were tested for their effects on cyclic AMP. In parallel studies, the inhibition of [^{125}I]iodohydroxybenzylpindolol (IHYP) binding to pineal β-adrenergic receptors by these compounds was studied. All of the agonists inhibited IHYP binding and elevated intracellular cyclic AMP in a dose-dependent fashion. The relative potencies of norepinephrine and its fluorine derivatives in both studies were found to be 5F-NE > NE > 2F-NE > 6F-NE. Increases in cyclic AMP were stereospecifically blocked by (−)-propanolol. From these studies it appears that fluorine substitution on NE at the 5-position enhances, and at the 2- and 6-position reduces, the ability to elevate cyclic AMP, as compared to that of norepinephrine, by altering agonist affinity for the β receptor. Additionally, subtle differences in the effects of the different fluorinated analogs on pinealocytes in the presence of weak antagonists were detected. In studies of antagonist action on agonist-induced cyclic AMP accumulation, substitution at the 5- or 6-position resulted in derivatives which, when compared with NE and 2F-NE, appeared less affected by the weak β-adrenergic antagonist (+)-propanolol and more affected by the α-adrenergic antagonist phentolamine.

Fluorine substitution of hydrogens in biologically important molecules provides an interesting and potentially valuable probe for the investigation of the electronic variables involved in biological responses. Fluorine, with a van der Waals radius close to that of hydrogen, imposes little steric alteration in the molecule. However, being highly electronegative, fluorine can considerably alter the electron distribution of the molecule [1]. The consequences of these effects are of interest for the understanding of the molecular interactions of molecules such as catecholamines, wherein phenolic hydroxyl ionization and possible side chain conformations could reflect altered electronic properties of the aromatic ring.

Fluorine substitution of the norepinephrine (NE)† ring has recently been reported to have distinct effects on the action of this compound [2–5]. Substitution at the 5-position (5-fluoronorepinephrine, 5F-NE) enhanced β-adrenergic activity. Substitution at the 2-position (2-fluoronorepinephrine, 2F-NE) appeared to leave β-adrenergic agonist activity unchanged while abolishing α-adrenergic agonist activity. In contrast, substitution at the 6-position (6-fluoronorepinephrine, 6F-NE) reduced the β-adrenergic while preserving the α-adrenergic activity of NE. Preliminary evidence, obtained using model systems capable of both α- and β-adrenergic responses, suggested that these effects were due to changes in the specificity of receptor binding.

To continue investigation of the effects of fluorine substitution, we decided to test the fluoronorepinephrines in a sensitive, well-defined system in which receptor binding and the resultant intracellular changes were easily measured. Intact neonatal rat pinealocytes [6] were chosen because, in this neuroendocrine tissue, intracellular cyclic AMP levels are under β-adrenergic control [7, 8], and intact cells may be used in direct receptor binding studies [9]. Using intact cells, it was possible to relate receptor

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† Abbreviations: NE, norepinephrine; cyclic AMP, cyclic adenosine 3′,5′-monophosphate; 5F-NE, 5-fluoronorepinephrine; 2F-NE, 2-fluoronorepinephrine; 6F-NE, 6-fluoronorepinephrine; DMEM, Dulbecco's Modified Eagle's Medium; FCS, fetal calf serum; HEPES, *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; and IHYP, [^{125}I]iodohydroxybenzylpindolol.

binding to intracellular effects and to test the hypothesis that alterations in the agonist effects of NE produced by fluorine substitution reflect changes in binding to β-adrenergic receptors.

MATERIALS AND METHODS

The sources or synthesis of chemicals used in these studies and the methods employed have been described recently in detail [3, 6, 9]. Briefly, pinealocytes are obtained by enzymatic and mechanical dissociation of pineal glands obtained from 2-day-old Sprague–Dawley rats (Zivic-Miller Co., Allison Park, PA). Cells are incubated in culture medium (DMEM with 10% FCS, penicillin 100 units/ml and streptomycin 100 μg/ml) for 24 hr prior to testing. They remain dispersed when incubated in bulk in large flasks and aggregate when incubated in 1.5-ml conical microtubes. After 24 hr cells in microtubes were directly stimulated. Cells in flasks were resuspended in assay medium (DMEM with 25 mM HEPES and ascorbic acid 100 μg/ml, pH 7.4) prior to stimulation or use in binding studies. Agonists were freshly diluted in 0.01 N HCl from stock solutions immediately prior to addition. After stimulation, the cells were centrifuged, the pellets were placed on dry ice, the cells were lysed with 5% PCA, and the resultant supernatant fractions were neutralized and assayed for cyclic AMP by radioimmunoassay according to published methods [10, 11]. [^{125}I]Iodohydroxybenzylpindolol (IHYP) binding to intact cells was performed in assay media by the addition of IHYP with or without a competing ligand followed by incubation. Cells were collected and washed on glass fiber filter papers according to published methods [9]. Non-specific binding was determined in the presence of 0.1 μM hydroxybenzylpindolol. Specific binding is the difference between total and non-specific binding. Proteins were measured by a dye binding technique [12].

The K_a values assigned to the agonists represent the concentration of the agonist that caused half-maximum cyclic AMP accumulation. The ID_{50} value represents the concentration of agonist that inhibited 50 per cent of specific IHYP binding in the same cell preparation. K_a values are computer generated by fitting the dose–response data with a four-parameter logistic equation in which one parameter defines the half-maximum response [9]. ID_{50} Values were determined from the midpoint of logit log transformations of the binding data.

RESULTS

Effects of fluorine substitution on the norepinephrine-stimulated elevation of cyclic AMP. To test the effects of fluorine substitution on the capacity of NE to elevate cyclic AMP in pinealocytes, cells were incubated with NE or one of the fluoronorepinephrines (Fig. 1A). Treatment with each agonist resulted in a dose-dependent increase in the accumulation of cyclic AMP with maximum accumulations approximately 20-fold greater than basal levels. The relative potencies of the agonists were 5F-NE ≥ NE > 2F-NE > 6F-NE. The maximum accumulation produced by each agonist was similar. These studies were conducted in the presence of complete culture medium. Since binding studies with IHYP are performed routinely in the absence of added protein, cyclic AMP accumulation was also measured after the cells had been resuspended in assay medium under the same conditions used in the binding studies (Fig. 1B). Under these conditions, the response to NE and the fluoronorepinephrines was qualitatively similar although the magnitude of the maximum cyclic AMP accumulation was about 75 per cent lower. We believe the difference could reflect the effects of media components on responsiveness or on cyclic AMP efflux. In these studies, (−)-NE was used rather than the racemic mixture.

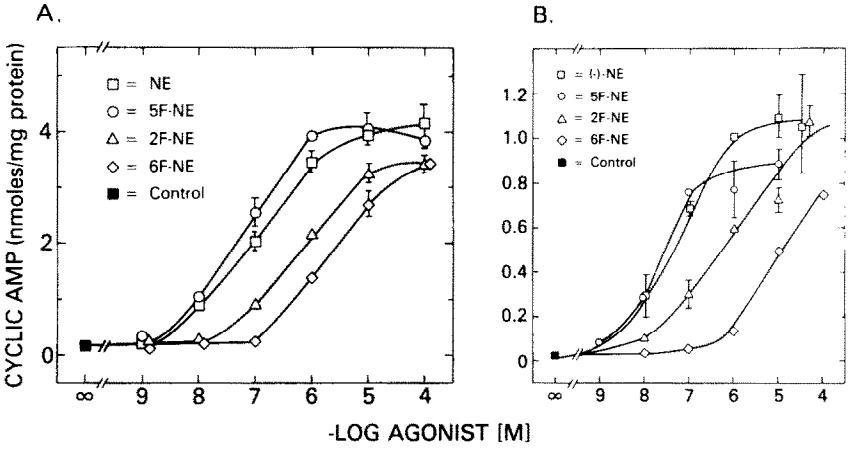

Fig. 1. Agonist-stimulated cyclic AMP accumulation in pinealocytes. Cell preparation is described in Materials and Methods. After 24 hr in culture, cells were (A) treated without additional manipulation (200,000 cells/0.5 ml) or (B) resuspended in assay medium (170,000 cells/0.5 ml), incubated for 20 min at 37°, and then treated with agonists. Cells were treated with agonists for 15 min at 37° and then pelleted. Intracellular cyclic AMP was assayed by radioimmunoassay. Agonists were added from solutions 10-fold more concentrated than final assay concentrations. Each point is the mean of duplicate determinations of four (A) or two (B) samples treated with the same agonist concentrations. Racemic mixtures of NE and the F-NEs were used except for the use of (−)-NE in (B). Bars represent S.E.M. Abbreviations: NE, norepinephrine; 5F-NE, 5-fluoronorepinephrine; 2F-NE, 2-fluoronorepinephrine; and 6F-NE, 6-fluoronorepinephrine.

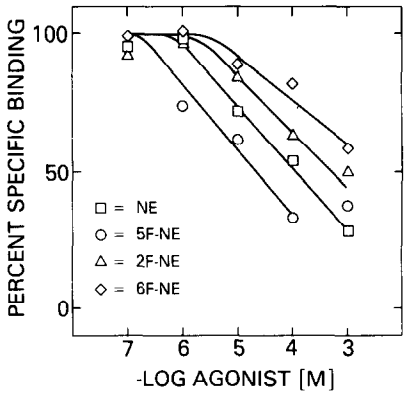

Fig. 2. Inhibition of IHYP binding to pinealocytes. Cells were prepared according to procedures described in Materials and Methods and procedure B in the legend to Fig. 1. Binding was initiated by the addition of agonist and IHYP. The final assay volume was 0.5 ml; the final IHYP concentration was 83 pM (150,000 cpm/tube). After 30 min at 37°, triplicate 100 μl samples were collected, filtered, and washed, and bound counts were determined. Non-specific binding was determined in the presence of 0.1 μM HYP. Racemic mixtures of all agonists were used. Points are means of triplicate determinations at each concentration expressed as per cent of specific binding in the absence of added agonist. S.E.M. was less than 10 per cent for each point. Abbreviations are as in Fig. 1.

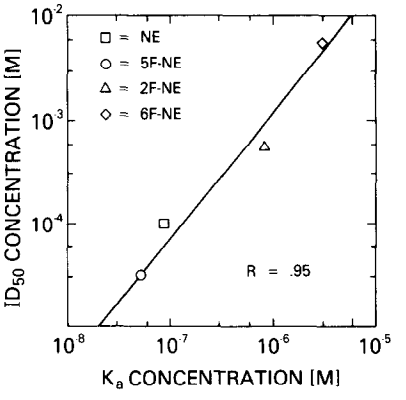

Fig. 3. Comparison of cyclic AMP accumulation with relative agonist affinities. Concentrations of each agonist giving 50 per cent of the maximum cyclic AMP response (K_a), calculated according to Methods from data in Fig. 1A, are plotted on the abscissa. Concentrations of each agonist giving 50 per cent inhibition of specific IHYP binding (ID_{50}), calculated according to procedures described in Materials and Methods, are plotted on the ordinate. The linear regression line was computer fit, R = 0.95. Abbreviations are as the legend to Fig. 1.

Effects of fluorine substitution on the inhibition of IHYP binding to pineal β-adrenergic receptors. The relative affinities of norepinephrine and the fluoronorepinephrines for the β-adrenergic receptor were determined in competitive binding studies in which the inhibition of binding of the specific β-adrenergic radioligand IHYP was measured (Fig. 2). All of the agonists produced a dose-dependent inhibition of IHYP binding. The relative potencies of the agonists for the inhibition of IHYP binding, and thus their relative affinities for the β-adrenergic receptor, were 5F-NE > NE > 2F-NE ≫ 6F-NE. This is the same order as that demonstrated in the cyclic AMP studies.

Comparison of the concentration producing half-maximum cyclic AMP accumulation (K_a) and the concentrations inhibiting 50 per cent of specific IHYP binding (ID_{50}) are shown in Fig. 3. An excellent correlation between the K_a and ID_{50} values for the fluoronorepinephrines and NE was obtained. The direction and magnitude of the change produced by fluorine substitution were the same when either parameter was considered. Comparison of the actual concentrations required to produce these effects reveals that all of the agonists were more potent in stimulating cyclic AMP accumulation than they were in inhibiting IHYP binding under the conditions used. This has been reported previously for NE in the dispersed pinealocyte preparation [9].

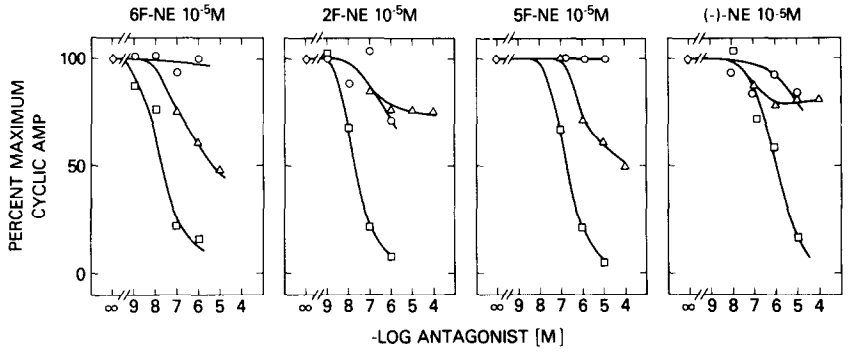

Fig. 4. Inhibition of agonist-stimulated cyclic AMP accumulation. Cells were prepared according to procedures described in Materials and Methods and the legend to Fig. 1B. Cells were treated for 15 min at 38° with 10 μM of each of the agonists in the absence or presence of (−)-propranolol (□—□), (+)-propranolol, (○—○), or phentolamine, (△—△). Abbreviations for agonists are as in the legend to Fig. 1. (−)-NE was used; the other agonists were racemic mixtures. Cyclic AMP was determined by radioimmunoassay. Means ± S.E.M. of duplicate determinations of two to three samples at each concentration were made. S.E.M. values were less than 20 per cent of the means. Because data were taken from multiple experiments, means were expressed as per cent of the mean maximum cyclic AMP stimulated by 10 μM agonist in a given experiment.

Effects of (−)- and (+)-propranolol on the elevation of cyclic AMP by the fluoronorepinephrines. To determine the effectiveness of β-adrenergic blockade on the stimulation of cyclic AMP by NE and the fluoronorepinephrines, cells were treated with either (+)- or (−)-propranolol and an agonist. (−)-Propranolol blocked the elevation of cyclic AMP produced by each agonist by at least 85 per cent (Fig. 4). The concentration of (−)-propranolol required to block the cyclic AMP response to each agonist (10 μM) by 50 per cent was least for 6F-NE, slightly greater for 2F-NE, and greater still for 5F-NE and NE, in agreement with the potency order seen for cyclic AMP accumulation. The somewhat higher concentration of propranolol needed to inhibit the response to NE compared to 5F-NE reflects the use of (−)-NE in these studies rather than the racemic mixture.

In contrast, (+)-propranolol appeared to have one of two effects. When used with 2F-NE or NE the concentration of (+)-propranolol required to inhibit cyclic AMP accumulation, as expected, was approximately 100-fold greater than that of (−)-propranolol. Under similar conditions, however, (+)-propranolol was essentially without effect on the stimulation of cyclic AMP accumulation by 5F-NE or 6F-NE, even at concentrations 1000-fold greater than (−)-propranolol.

Effects of phentolamine on the elevation of cyclic AMP by the fluoronorepinephrines. High concentrations of the α-adrenergic antagonist phentolamine have been shown previously to inhibit specific IHYP binding to pinealocytes [9], presumably through a weak interaction with β-adrenergic receptors. For this reason the effect of phentolamine on the stimulation of cyclic AMP by NE and the fluoronorepinephrines was also determined. Phentolamine partially blocked the stimulation of cyclic AMP accumulation by each agonist (Fig. 4). The effect of phentolamine was most marked in the antagonism of the stimulation of cyclic AMP accumulation by 6F-NE and 5F-NE. In these cases, phentolamine at a concentration of 1 or 10 μM produced about a 50 per cent decrease in the maximum accumulation of cyclic AMP. In contrast, with 2F-NE and NE only a 20–25 per cent reduction of cyclic AMP accumulation could be realized at phentolamine concentrations of 100 μM. Furthermore, this effect of phentolamine on the stimulation of cyclic AMP formation by 2F-NE and NE did not appear to be dose dependent at higher phentolamine concentrations.

DISCUSSION

The results presented in this report provide additional evidence that substitution of fluorine on the aromatic ring of norepinephrine yields derivatives with biological activities distinct from those of the parent compound. The various positions of the fluorine substituents conferred marked differences in the apparent affinities of these norepinephrine analogs for β-adrenergic receptors and, as a consequence, altered the stimulation of cyclic AMP accumulation. In addition, there appear to have been some changes in the inhibitory effects observed when (+)-propranolol or phentolamine was used to block agonist-stimulated cyclic AMP accumulation.

Before discussing these effects in detail, it is valuable to review some of the characteristics of the experimental system used. Intact neonatal rat pinealocytes appear to share all the characteristics of postsynaptic adrenergic regulation of adenylate cyclase seen in adult pineal glands and are free from the pre-synaptic elements, including endogenous norepinephrine which might make interpretation of the results more difficult [6–8]. Most important, it appears that the stimulation of increases in cyclic AMP in pinealocytes is under the sole control of β-adrenergic receptors. Although α-adrenergic agonists, such as phenylephrine, are also effective in increasing cyclic AMP in this system [6, 9], this effect is stereospecifically blocked by (−)-propranolol [9] and appears to be mediated entirely by the interaction of the agonist with β-adrenergic receptors. An important property of this system for comparison of the effects of fluorine-substituted analogs with those of NE is the direct delivery of the agonist to the cell surface, unaffected by diffusion or transport through the tissue. Thus, except for considerations of the microenvironment of the receptor, all of the agonists should have had similar, direct access to the receptors.

In the present study, it was found that fluorine substitution of NE resulted in either a small enhancement or a significant reduction in the capacity to elevate cyclic AMP. The change depended on the position of the fluorine. The order of potencies observed for cyclic AMP stimulation was 5F-NE > NE > 2F-NE > 6F-NE. Maximum cyclic AMP accumulations produced by the analogs were similar to that of NE. Fluorine substitution appeared to shift the response curves parallel to that of NE. This suggested that the intrinsic activities of the fluoronorepinephrines were similar to that of NE but that their affinities for the β-adrenergic receptor were altered. This conclusion was supported by direct analysis of the relative abilities of the agonists to inhibit IHYP binding. The observed alterations in receptor binding were in the same direction and of a magnitude similar to that of cyclic AMP effects for each of the fluoronorepinephrines. The correlation between the concentrations resulting in half-maximum effects was excellent. The difference in the absolute concentrations required for the half-maximum effect in the binding studies compared with the cyclic AMP studies is a result of using intact cells rather than membrane or broken cell preparations [9, 13]. Thus, in this system, the major effect of fluorine substitution appears to be an alteration of the affinity of NE for β-adrenergic receptors.

The findings in the present study differ slightly from those reported previously. In studies with the guinea pig atrial preparation, norepinephrine and 2F-NE were found to have equipotent β-agonist activity [2–4]. The same was true in studies of rat cerebral cortical slice cyclic AMP accumulation [3, 5]. In the latter studies, however, there was some discrepancy between the rank order of potencies for cyclic AMP stimulation and the binding of the fluoronorepinephrines to rat cerebral cortical membrane β-adrenergic receptors [3]. In the pineal system 2F-

NE is less potent than norepinephrine, and there is good correlation between its binding and cyclic AMP effects. The cause for these differences with previous studies is not clear. Perhaps the homogenous nature of the cells in the pineal gland and their responsiveness only to β-adrenergic stimulation are important factors. Even though the cortical slice studies were performed using selective blockers, differences in receptor distribution, the presence of α- and β-adrenergic responsiveness, and the diversity of tissue elements may introduce factors that complicate the interpretation of results. Additionally, tissue-specific differences in the behaviors of β-adrenergic agonists may also influence experimental results [13].

The action of (−)-propranolol in blocking essentially all of the increases in cyclic AMP stimulated by norepinephrine and the fluoronorepinephrines is consistent with the established β-adrenergic control of cyclic AMP in pinealocytes. In previous reports, 6F-NE was demonstrated to have lost essentially all of its β-adrenergic activity but to have retained its α-adrenergic activity [2–5]. Although it was not possible in pinealocytes to investigate meaningfully the α-adrenergic potency of 6F-NE in terms of cyclic AMP response, it is possible to use pinealocytes to assess α-adrenergic effects by measuring ^{32}P-incorporation into membrane phospholipids, a cyclic AMP independent effect mediated by pineal α-adrenergic receptors [14, 15].

In addition to the major finding of alteration of norepinephrine affinity for the β receptor, a curious additional finding was obtained. (+)-Propranolol showed some limited antagonism of cyclic AMP accumulation when stimulated by NE and 2F-NE but was totally ineffective when used with 6F-NE or 5F-NE. In contrast, phentolamine was more potent in blocking stimulation of cyclic AMP by 6F-NE and 5F-NE than by NE or 2F-NE. We have no simple explanation for these findings and will postpone further comment until more experimental data are available.

The demonstration of distinct differences in the behaviors of the fluoronorepinephrines raises questions concerning the mechanism by which fluorine substitution alters the affinity for β-adrenergic receptors. The introduction of a fluorine on the ring alters the pK_a of norepinephrine. Reported pK_a values for NE, 6F-NE, 2F-NE and 5F-NE are 8.9, 8.5, 7.8 and 7.9 respectively [2]. With respect to pK_a, NE and 6F-NE are most similar. In the present studies, however, NE and 6F-NE had the most disparate behavior, suggesting that different pK_a is not the basis for the observed effects and that perhaps alterations in groups other than on the ring might be of importance. A full understanding of the molecular basis of the observed effects of fluorination of the norepinephrine ring awaits further studies.

REFERENCES

1. P. Goldman, *Science* **164**, 1123 (1969)
2. D. Cantacuzene, K. L. Kirk, D. H. McCulloh and C. R. Creveling, *Science* **204**, 1217 (1979)
3. K. L. Kirk, D. Cantacuzene, Y. Nimitkitpaisan, D. McCulloh, W. L. Padgett, J. W. Daly and C. R. Creveling, *J. med. Chem.* **22**, 1493 (1979).
4. C. R. Creveling, D. H. McCulloh, D. Cantacuzene and K. L. Kirk, *Fedn Proc.* **38** (3), 251 (1979).
5. J. W. Daly, W. Padgett, Y. Nimitkitpaisan, D. Cantacuzene, K. L. Kirk and C. R. Creveling, *Fedn Proc.* **38** (3), 533 (1979).
6. M. Buda and D. C. Klein, *Endocrinology* **103** (4), 1483 (1978).
7. D. C. Klein, in *The Hypothalamus* (Eds. S. Reichlin, R. J. Baldessarini and J. B. Martin), p. 303. Raven Press, New York (1978).
8. D. C. Klein, M. J. Buda, C. L. Kapoor and G. Krishna, *Science* **199**, 309 (1978).
9. D. A. Auerbach, D. C. Klein, C. Woodard and G. D. Aurbach, *Endocrinology*, **108**, 559 (1981).
10. J. F. Harper and G. Brooker, *J. Cyclic Nucleotide Res.* **1**, 207 (1975).
11. E. K. Frandsen and G. Krishna, *Life Sci.* **18**, 529 (1976).
12. M. M. Bradford, *Analyt. Biochem.* **72**, 660 (1976).
13. M. E. Maguire, E. M. Ross and A. G. Gilman, in *Advances in Cyclic Nucleotide Research*, Vol. 8 (Eds. P. Greengard and G. A. Robinson), p. 1. Raven Press, New York (1977).
14. T. L. Smith, J. Eichberg and G. Hauser, *Life Sci.* **24**, 2179 (1979).
15. J. Eichberg, H. M. Shein, M. Schwartz and G. Hauser, *J. biol. Chem.* **248**, 3615 (1973).