Biochimica et Biophysica Acta, 334 (1974) 218–225

BBA 67102

POTENT MAGNESIUM-DEPENDENT INHIBITION OF ADENYLATE CYCLASE ACTIVITY FROM GUINEA PIG LUNG BY ADENOSINE AND OTHER 9-SUBSTITUTED ADENINES

IRA WEINRYB and INGE M. MICHEL

Department of Biochemical Pharmacology, Squibb Institute for Medical Research, Princeton, N.J. 08540 (U.S.A.)

(Received August 6th, 1973)

SUMMARY

The inhibition of adenylate cyclase activity (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) from guinea pig lung by adenosine and a number of 9-substituted adenines was Mg^{2+}-dependent, the compounds being up to 13 times more potent at saturating or near saturating Mg^{2+} concentrations (11.8 mM) than at limiting (1.8 mM) concentrations. Inhibition by adenine and 6-mercaptopurine did not show a Mg^{2+} dependence. The most potent inhibitors of cyclase activity at 11.8 mM Mg^{2+} of the 9-substituted adenines tested were: 9-(tetrahydro-5-methyl-2-furyl)adenine ($I_{50} = 8\,\mu M$), 9-(tetrahydro-2-furyl)adenine ($I_{50} = 10\,\mu M$), 9-cyclopentyladenine ($I_{50} = 20\,\mu M$), and 9-furfuryladenine ($I_{50} = 26\,\mu M$). The inhibition of lung cyclase activity by 9-(tetrahydro-2-furyl)adenine was deduced to be hyperbolic non-competitive at 1.8 mM ($K_i = 1.2 \cdot 10^{-4}$ M) and 11.8 mM ($K_i = 2.5 \cdot 10^{-6}$ M) Mg^{2+} from analysis of double-reciprocal plots; these plots showed a concave downward non-linearity in the presence of inhibitor at both Mg^{2+} concentrations. This non-linearity was eliminated under conditions where Mg^{2+} levels were never in excess of those of ATP. Hill plots of the inhibition by 9-(tetrahydro-2-furyl)adenine (and by adenosine) suggested that negative co-operativity is involved in binding to the enzyme. The possibility that the Mg^{2+}-dependence of inhibitory potency of adenosine and its analogs functions as a cellular control mechanism is discussed.

INTRODUCTION

In a previous communication from this laboratory [1], adenosine was shown to inhibit adenylate cyclase activity (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) from guinea pig lung; inhibition by 50% was achieved at a concentration of approx. 2 mM. Less than saturating Mg^{2+} concentrations [1] were used in the assay of this inhibitory activity, since the available data [2, 3] indicated that adenine nucleosides did not bind Mg^{2+}. More recent data [4] supports this point. However, a subsequent examination of the inhibitory potency of adenosine revealed a strong and novel

dependence on Mg^{2+} concentration. This dependence was also observed for selected adenosine analogs, many of which were very potent inhibitors of cyclase activity from guinea pig lung. These observations, and their implications, are the subject of this report.

METHODS

Enzyme preparation

Tissue was obtained from male guinea pigs that had been stunned and decapitated. Lung tissue was combed away from the excised tracheobronchial tree, and a cyclase fraction was prepared as previously described [1, 5].

Assay of adenylate cyclase activity

Assay in duplicate of adenylate cyclase activity was accomplished as previously reported [1, 5], with [α-^{32}P]ATP as substrate and an isolation procedure for cyclic AMP that included the use of cyclic [^{3}H]AMP as a recovery standard, Dowex 50 chromatography, and $BaSO_4$ treatment. The basic assay system included 1.8 mM $MgCl_2$, 0.8 mM glycylglycine, 32 mM Tris (pH 7.8), 1.2 mM ATP, $3 \cdot 10^6$–$5 \cdot 10^6$ cpm [α-^{32}P]ATP, (50 μl) particulate enzyme fraction (100–250 μg enzyme protein), 1 mM cyclic AMP, and test compound (if any) in a total volume of 0.59 ml. Incubation times of 5 min (instead of the routine 15-min period) were used in the kinetic studies to ensure linear reaction rates [1], and this sometimes necessitated the use of larger amounts of [α-^{32}P]ATP (up to $12 \cdot 10^6$ cpm/assay) than formerly employed. The experiments reported here were carried out on five different enzyme preparations, each of which was split into small aliquots and stored under liquid nitrogen until use, as outlined previously [1, 5, 6].

Concentrations of inhibitor necessary to lower cyclase activity to 50% of control values (I_{50}) were determined in the presence of total Mg^{2+} concentrations of 11.8 mM and, in some cases, 1.8 mM. The former Mg^{2+} concentration is often saturating for lung cyclase fractions [1], although, for the preparation used in the kinetic studies below (Figs 3–5), activity at 11.8 mM Mg^{2+} was about 80% of the maximum activity, estimated from a double-reciprocal plot of the Mg^{2+}–activity relationship.

MATERIALS

Standard assay reagents were obtained as previously described [5, 6]. Adenine, adenosine, 2′-deoxyadenosine, and 3′-deoxyadenosine were purchased from Sigma. 9-Cyclohexyladenine and 6-mercaptopurine were obtained from Heterocyclic Chemical and Burroughs Wellcome, respectively. 9-Cyclopentyladenine, 9-(5-methyl-2-furyl)adenine, 9-(tetrahydro-5-methyl-2-furyl)adenine, and 9-(tetrahydro-2-furyl)-adenine were gifts from Dr M. Zedeck (Sloan-Kettering). 9-Cyclopentyl-6-chloro-8-azapurine was a gift from Dr R. W. Ruddon (University of Michigan). The remaining adenosine analogs were supplied by Miss K. A. Losee of this Institute. Sparingly soluble compounds were homogenized in the assay buffer to form fine suspensions at high concentrations and were diluted appropriately. All of the compounds appeared soluble at concentrations of 1 mM or less.

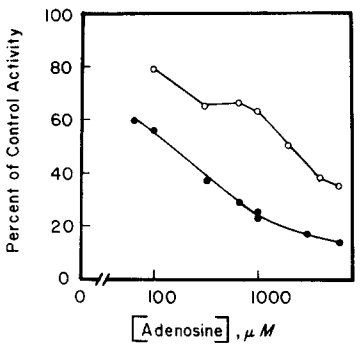


Fig. 1. Inhibition of adenylate cyclase activity from guinea pig lung by adenosine in the presence of 1.8 mM (○) and 11.8 mM (●) Mg^{2+}. In the absence of adenosine, cyclase activity was 1029 pmoles cyclic AMP/mg protein per 15 min at 1.8 mM Mg^{2+}, and 2841 pmoles cyclic AMP/mg protein per 15 min at 11.8 mM Mg^{2+}.

RESULTS

The inhibition of cyclase activity from guinea pig lung by adenosine is detailed in Fig. 1 and summarized in Table I. Whereas cyclase activity was at least doubled at saturating or near saturating Mg^{2+} concentrations (11.8 mM) relative to limiting concentrations (1.8 mM) (see ref. 1 and legend to Fig. 1), the potency of inhibition of adenosine was increased approx. 10-fold. Adenine was a much weaker inhibitor at

TABLE I

EFFECTS OF 9-SUBSTITUTED PURINES ON BASAL LUNG ADENYLATE CYCLASE ACTIVITY

Compound	I_{50} (mM) for compound assayed in presence of	
	1.8 mM Mg^{2+}	11.8 mM Mg^{2+}
Adenine	>6.0 (41% inhibition at 6 mM)	>6.0 (47% inhibition at 6 mM)
Adenosine	2.0	0.15
2′-Deoxyadenosine	—	0.025, 0.035
3′-Deoxyadenosine	0.55	0.06
9-(Tetrahydro-2-furyl)adenine	0.1	0.01
9-(Tetrahydro-5-methyl-2-furyl)adenine	—	0.008
9-(5-Methyl-2-furyl)adenine	—	3.4
9-Furfuryladenine	0.3	0.026
4-(9-Adenyl)-2,3-dihydroxybutyrate	0.22	0.06
9-Phenyladenine	>6.0	6.0
9-Cyclohexyladenine	—	0.058
9-Cyclopentyladenine	0.10	0.020
9-Benzyladenine	2.0, 1.8	0.2
2,6-Diaminopurine	—	>6.0
9-Benzyl-2,6-diaminopurine	—	>6.0
9-Furfuryl-8-aza-adenine	—	2.8
9-Cyclopentyl-6-chloro-8-azapurine	—	1.7

the lower Mg^{2+} concentration, and did not show Mg^{2+}-dependent inhibition (Table I), nor did 6-mercaptopurine, which gave I_{50} values of 0.4 and 0.6 mM at 1.8 mM Mg^{2+} and of 0.4 mM at 11.8 mM Mg^{2+}.

Inhibition of cyclase activity by a number of 9-substituted purines besides adenosine was also examined, and all of those studied showed Mg^{2+}-dependent inhibitory potencies (Table I). Increases in inhibitory potencies ranged from a 10-fold increase at the higher Mg^{2+} concentration (3′-deoxyadenosine, 9-(tetrahydro-2-furyl)adenine, 9-furfuryladenine, and 9-benzyladenine) to 3–5-fold increases for 4-(9-adenyl)-2,3-dihydroxybutyrate and 9-cyclopentyladenine. Typical plots of inhibition vs concentration at both Mg^{2+} concentrations are shown for 9-(tetrahydro-2-furyl)adenine (Fig. 2).

Many of the 9-substituted adenines tested were more potent than adenosine as inhibitors of lung cyclase activity at either Mg^{2+} concentration. Thus, 2′- and 3′-deoxyadenosine were, respectively, 5 and 2.5 times as potent as adenosine at high Mg^{2+} concentrations. The tetrahydrofuryladenines were extremely potent inhibitors;

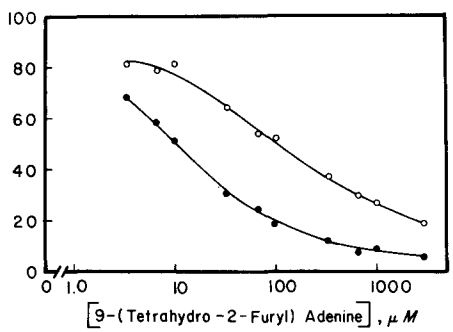


Fig. 2. Inhibition of adenylate cyclase activity from guinea pig lung by 9-(tetrahydro-2-furyl)adenine in the presence of 1.8 mM (○) and 11.8 mM (●) Mg^{2+}. In the absence of inhibitor, cyclase activity was 721 pmoles cyclic AMP/mg protein per 15 min at 1.8 mM Mg^{2+}, and 2481 pmoles cyclic AMP/mg protein per 15 min at 11.8 mM Mg^{2+}. Ordinate: Percent of control activity.

9-(tetrahydro-2-furyl)adenine and 9-(tetrahydro-5-methyl-2-furyl)adenine were 15–20 times as potent as adenosine at 11.8 mM Mg^{2+}. Inhibition by 4-(9-adenyl)-2,3-dihydroxybutyrate was equivalent to that by 3′-deoxyadenosine at high Mg^{2+} concentrations, and was surprisingly effective, considering the presence of an acyclic substituent at the 9-position. Carbocyclic substitutions at the 9-position gave variable results. 9-Phenyladenine was relatively inactive at 11.8 mM Mg^{2+}, while the 9-benzyl derivative was equipotent with adenosine. The 2-amino derivative of 9-benzyladenine, however, is a much less potent inhibitor than the parent compound. The 9-cyclohexyl and 9-cyclopentyl derivatives were, respectively, 2.5 and 7.5 times as effective as adenosine as cyclase inhibitors. Another 9-substituted adenine more powerful than adenosine as an inhibitor was 9-furfuryladenine, which was equipotent with 2′-deoxyadenosine.

The most striking difference in inhibitory effectiveness between closely related structures was that between 9-(5-methyl-2-furyl)adenine and 9-(tetrahydro-5-methyl-2-furyl)adenine. Saturation of the furan ring resulted in a 425-fold increase in potency as a cyclase inhibitor.

Some differences in relative potencies of inhibition by the adenosine analogs at the two Mg^{2+} concentrations are manifest in Table I. Thus, 3'-deoxyadenosine, 4-(9-adenyl)-2,3-dihydroxybutyrate and 9-cyclopentyladenine were more effective inhibitors relative to adenosine at low than at high Mg^{2+} concentrations. The 9-cyclopentyl derivative, in particular, was 20 times as potent as adenosine at 1.8 mM Mg^{2+}, but only 7.5 times as potent at 11.8 mM Mg^{2+}.

Kinetic studies with 9-(tetrahydro-2-furyl)adenine as a model adenosine analog were carried out. Fig. 3 shows a double-reciprocal plot of the activity of lung adenylate cyclase in the absence and presence of the adenosine analog as inhibitor at a Mg^{2+} concentration of 1.8 mM. Fig. 4 is a similar plot for a Mg^{2+} concentration of 11.8 mM. The K_m values for ATP of 0.25 and 0.16 mM, respectively, are consistent with values

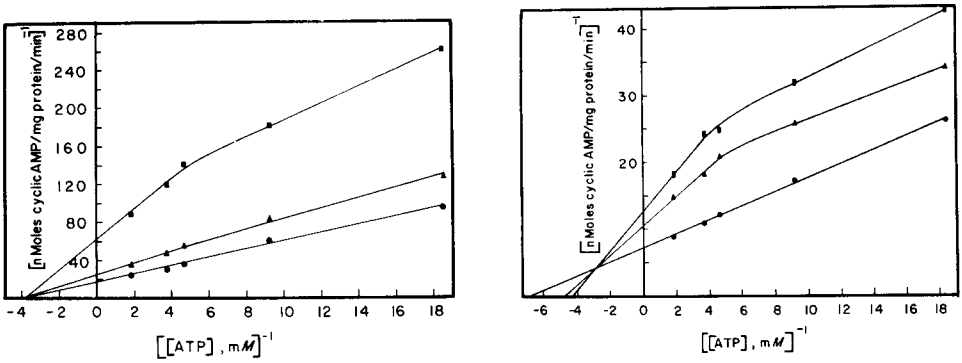


Fig. 3. Double-reciprocal plot of the kinetics of lung cyclase activity at 1.8 mM Mg^{2+} in the absence of inhibitor (●) and in the presence of $2 \cdot 10^{-5}$ M (▲) and $5 \cdot 10^{-4}$ M (■) 9-(tetrahydro-2-furyl)adenine.

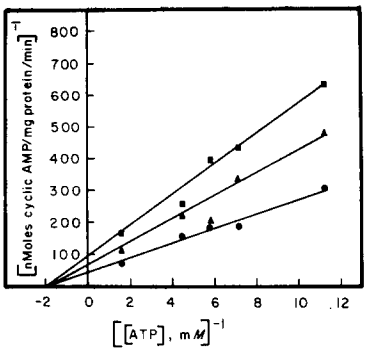
Fig. 4. Double-reciprocal plot of the kinetics of lung cyclase activity at 11.8 mM Mg^{2+} in the absence of inhibitor (●) and in the presence of $3 \cdot 10^{-6}$ M (▲) and $3 \cdot 10^{-5}$ M (■) 9-(tetrahydro-2-furyl)adenine.

previously reported by us of 0.17, 0.21, and 0.32 mM for various preparations of cyclase from guinea pig lung [1]. For both Mg^{2+} concentrations, the inhibition of cyclase activity by the adenosine analog appeared to be non-competitive with respect to substrate (ATP), as defined by Cleland [7]. The variation of intercepts with inhibitor concentration for the data of Figs 3 and 4 was compatible with hyperbolic non-competitive inhibition [7], and yielded K_i values of $1.2 \cdot 10^{-4}$ and $2.5 \cdot 10^{-6}$ M for Mg^{2+} concentrations of 1.8 and 11.8 mM, respectively*.

A feature of the plots of Figs 3 and 4 is the non-linearity observed in the presence of inhibitor. This non-linearity is eliminated under conditions where all the Mg^{2+} present is complexed with ATP; i.e. at a Mg^{2+}:ATP ratio of 0.95, for all ATP concentrations used (Fig. 5). The inhibition was again hyperbolic non-competitive, and the K_i value was $2.4 \cdot 10^{-5}$ M.

When the data of Fig. 2 were replotted according to a Hill-type relationship

* The kinetic behavior depicted in Fig. 4 is not totally hyperbolic non-competitive, since the intercepts, but not the slopes, of the double-reciprocal plots are hyperbolic functions of the inhibitor concentration.



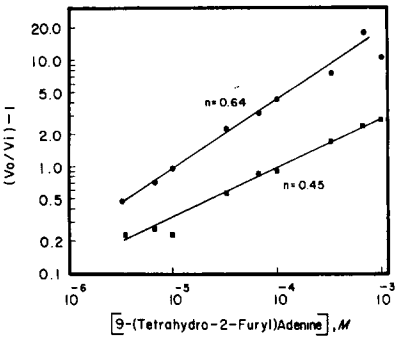


Fig. 5. Double-reciprocal plot of the kinetics of lung cyclase activity at a constant Mg^{2+}:ATP ratio equal to 0.95 in the absence of inhibitor (●) and in the presence of $2 \cdot 10^{-5}$ M (▲) and $5 \cdot 10^{-4}$ M (■) 9-(tetrahydro-2-furyl)adenine.

Fig. 6. Hill plot of the inhibition of lung cyclase activity by 9-(tetrahydro-2-furyl)adenine at 1.8 mM (■) and 11.8 mM (●) Mg^{2+} (data of Fig. 2). V_0 and V_i are the specific activities of lung cyclase (pmoles cyclic AMP/mg protein per 15 min) in the absence and presence of i M inhibitor, respectively.

[8, 9], the Hill coefficients* at either 1.8 or 11.8 mM Mg^{2+} were less than 1, suggesting that the binding of inhibitor may be negatively co-operative [9] (Fig. 6). The Hill coefficients for the two Mg^{2+} concentrations were also significantly different. A similar replot of the data of Fig. 1 for adenosine (not shown) also showed Hill coefficients of less than 1, although there was little difference between the values at the two Mg^{2+} concentrations (n approx. 0.5 at either concentration).

DISCUSSION

These investigations have shown that several 9-substituted adenines are potent inhibitors of the basal adenylate cyclase activity from guinea pig lung and that these compounds interact with the enzyme in a complex manner that is dependent on the Mg^{2+} concentration. This marked Mg^{2+} dependence was not observed in the inhibition of cyclase activity by adenine and 6-mercaptopurine, two purines unsubstituted in the 9-position, nor has it been seen for inhibition by cyclic nucleotides, including 6-thiopurine cyclic ribotide [1] and 2′-*O*-palmitoyl cyclic AMP (unpublished experiments). The data for 9-(tetrahydro-2-furyl)adenine also indicates that the excess Mg^{2+}, interacting with the cyclase from guinea pig lung to stimulate its activity, may simultaneously also bind (perhaps at other sites) so as to change the affinity of inhibitor for the enzyme, as reflected in the variation of the K_i values with both absolute Mg^{2+} concentration and Mg^{2+}:ATP ratio. While this study was in progress, Fain et al. [10] published the results of experiments with several adenine nucleosides showing that these adenosine analogs were capable of inhibiting cyclase activity from adipocytes. Our experiments suggest that inhibition of cyclases by adenosine analogs

* The specific enzyme activities presented in Fig. 2 are the result of 15 min incubations and thus are not, strictly speaking, initial velocities. However, the use of the relative velocity parameter (V_0/V_i) leads to well-behaved plots that suggest the results are valid. Similar remarks apply to the replot of the data of Fig. 1.

is not restricted to the enzyme from fat cells, and that it is a property of adenines with a wide range of substituents in the 9-position.

Comparison of the inhibitory potency of adenosine with that of ADP and adenine had led us to conclude that most of the binding determinants for substrate reside in the nucleoside [1]. However, the discovery of non-competitive inhibition of lung cyclase activity by 9-(tetrahydro-2-furyl)adenine implies that this compound interacts with the enzyme at a locus or loci different in some respects from that at which substrate (ATP) is bound, and further suggests that adenosine and adenosine analogs in general may inhibit lung cyclase activity non-competitively as well. Indeed, McKenzie and Bär [11] have recently found that the inhibition of rat brain cyclase activity by adenosine is not competitive with respect to ATP. Adenosine and 9-substituted adenines have been shown to inhibit the binding of ATP non-competitively in the case of at least one other enzyme, tyrosyl transfer RNA synthetase from *Escherichia coli* [12].

The kinetic mechanism of inhibition of the model adenosine analog, as analyzed from the double-reciprocal plots, was, in all cases, hyperbolic non-competitive. This kind of inhibition can result from a combination of inhibitor with an enzyme–substrate complex which lowers the rate of (but does not eliminate) the formation of product [7]. Under certain conditions, such partial inhibition can produce curved double-reciprocal plots in the presence of inhibitor such as seen in Figs 3 and 4. Concave-downward double-reciprocal plots of this kind have also been regarded as diagnostic for negative co-operativity in the binding of effectors to enzymes [9].

Negative co-operativity has been described as a phenomenon whereby the binding of a particular small molecule to a macromolecule such as an enzyme induces conformational changes such that the affinity of additional molecules of this type for additional binding sites on the macromolecule is decreased [13, 14]. The apparent negative co-operativity observed for inhibition by 9-(tetrahydro-2-furyl)adenine implies multiple binding sites on the adenylate cyclase complex for this compound. The difference in the Hill coefficients at the two Mg^{2+} concentrations is an additional indication that the enzyme conformations under each condition are distinct with respect to the binding of this inhibitor. The lack of significant variation of the Hill coefficients with Mg^{2+} concentration for inhibition by adenosine may reflect subtle differences in the binding of these two 9-substituted purines to the enzyme. Negatively co-operative inhibition also predicts relatively shallow inhibition–concentration curves [9], that are, in fact, observed for both the model adenosine analog and for other adenine derivatives.

An alternative interpretation of the data presented here may exist*. If our cyclase preparations contained distinct cyclases, perhaps from different cell types, with differing Mg^{2+} concentration requirements, and if the enzyme requiring higher Mg^{2+} concentrations were more sensitive to inhibition by adenosine analogs, it might be possible to obtain results similar to those discussed above. Whereas this situation is possible, it is not highly likely, for it postulates a fortuitous coincidence of cyclases with strikingly different inhibitor and Mg^{2+} sensitivities.

If adenine nucleosides regulate cyclase activity in lung as has been suggested

* This interpretation was suggested by a referee.

by Fain et al. [10] for cyclase in the fat cell; i.e. as feedback inhibitors, then the effect of Mg^{2+} on the inhibition may be physiologically relevant, as well as biochemically interesting. Saturating concentrations of Mg^{2+} may be as low as 5–6 mM for lung cyclase fractions [1], and intracellular Mg^{2+} concentrations are likely to reach or exceed this level. Although data on Mg^{2+} concentrations in lung tissue are not, to our knowledge, available, it is known that Mg^{2+} levels in mammalian blood cells are as high as 6 mM, and that tissue levels of this cation, based on fresh weight, reach similar levels in liver, and substantially higher concentrations in kidney and brain [15]. We have shown that the responsiveness to isoproterenol of enzyme fractions from lung is reduced as the basal activity increases in the presence of increasing Mg^{2+} concentrations [1]. However, if cyclase activity in the cell is, under some conditions, controlled by Mg^{2+} concentrations rather than by hormone, the increased potency of inhibition of adenine nucleosides at higher Mg^{2+} concentrations might ensure against inappropriate synthesis of cyclic AMP as a result of transient increases in Mg^{2+} levels in the proximity of the cyclase catalytic unit.

The synthetic adenosine analogs, shown here to be generally more potent inhibitors of lung cyclase activity than adenosine, might be expected to show pharmacologic activity consistent with that of an adenylate cyclase inhibitor, in cells and tissues in which adenine nucleosides are effective.

ACKNOWLEDGMENT

The authors thank Dr S. M. Hess for continued interest and encouragement.

REFERENCES

1 Weinryb, I., Michel, I. M. and Hess, S. M. (1973) Arch. Biochem. Biophys. 154, 240–249
2 Martell, A. E. and Schwarzenbach, G. (1956) Helv. Chim. Acta 39, 653–661
3 Walaas, E. (1958) Acta Chem. Scand. 12, 528–536
4 Jordon, F. and McFarquhar, B. Y. (1972) J. Am. Chem. Soc. 94, 6557–6558
5 Weinryb, I., Michel, I. M., Alicino, J. and Hess, S. M. (1971) Arch. Biochem. Biophys. 146, 591–596
6 Weinryb, I. and Michel, I. M. (1971) Experientia 27, 1386
7 Cleland, W. W. (1963) Biochim. Biophys. Acta 67, 173–187
8 Atkinson, D. E. (1966) Annu. Rev. Biochem. 35, 85–124
9 Levitzki, A. and Koshland, Jr, D. E. (1969) Proc. Natl. Acad. Sci. U.S. 62, 1121–1128
10 Fain, J. N., Pointer, R. H. and Ward, W. F. (1972) J. Biol. Chem. 247, 6866–6872
11 McKenzie, S. G. and Bär, H. P. (1973) Can. J. Physiol. Pharmacol. 51, 190–196
12 Santi, D. V. and Peña, V. A. (1973) J. Med. Chem. 16, 273–280
13 Conway, A. and Koshland, Jr, D. E. (1968) Biochemistry 7, 4011–4022
14 Russell, T. R., Thompson, W. J., Schneider, F. W. and Appleman, M. M. (1972) Proc. Natl. Acad. Sci. U.S. 69, 1791–1795
15 Biochemists' Handbook (1961) (Long, C., ed.), Chapt. 4, pp. 637–918, E. and F. N. Spon, Ltd, London