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The NTP phosphate donor in kinase reactions: Is ATP a monopolist?*

David Shugar

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, A. Pawińskiego 5a, 02–106 Warsaw; Department of Biophysics, Institute of Experimental Physics, University of Warsaw, F. Żwirki i S. Wigury 93, 02–089 Warsaw, Poland

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This brief overview describes some of the properties of various cellular phosphotransferase systems, with particular emphasis on nucleoside 5′-triphosphate (NTP)-dependent protein kinases and nucleoside kinases, for which it is widely and implicitly assumed that ATP is the intracellular phosphate donor. Numerous examples are presented, based on the *in vitro* properties of these enzymes, to show that ATP is not the only, or frequently not even the major, phosphate donor, and that this is probably reflected *in vivo*. It is pointed out that *in vitro* studies of donor and acceptor specificities of kinases must take account of the intracellular concentrations of nucleoside 5′-triphosphates, a problem also relevant to the design of nucleoside analogues as chemotherapeutic agents. Attention is also drawn to NTP analogues as substrates/inhibitors of protein kinases, and to several examples of low-molecular mass non-peptide substrates of these enzymes.

Intracellular phosphorylation of a multitude of cellular constituents is a widespread phenomenon, catalyzed by enzymes denoted as phosphotransferases, many of which are further classified as kinases. A typical classical kinase is hexokinase, which catalyzes the reaction:

ATP + D-hexose →
→ ADP + D-hexose-6-phosphate

The term kinase originated from the Greek "kinein", to move or to transfer, and is now generally applied to enzymes which transfer the γ-phosphate of a nucleoside 5′-triphosphate (NTP, usually, but not always, ATP) to an acceptor such as a sugar, a lipid, a nucleoside, amino-acid residues of a protein (protein kinase, PK). The present review is devoted largely, but not exclusively, to nucleoside and protein kinases, with particular emphasis on the nature and role of the NTP phosphate donor. The provocative nature of the sub-title of this communication is intended simply to emphasize the fact that there are now sufficient documented instances where ATP is not the

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Abbreviations: N, nucleoside; NTP, nucleoside 5′-triphosphate; PK, protein kinase; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; PTK, protein-tyrosine kinase; CK-I and CK-II, previously known as casein kinases 1 and 2; TK1, cytosolic thymidine kinase; TK2, mitochondrial thymidine kinase; dCK, deoxycytidine kinase; dGK, deoxyguanosine kinase; PP_i, inorganic pyrophosphate; poly-P_i, inorganic polyphosphate; EGF, epidermal growth factor.

only, or even major, phosphate donor, and the resulting obvious need of considering the eventual biological consequences.

Nucleoside kinases catalyze the reaction:

$$ATP + N \rightarrow ADP + 5'\text{-}NMP,$$

where N is a nucleoside. This reaction is known as the salvage pathway for provision of nucleotide precursors for nucleic acid synthesis, and complements *de novo* synthesis of such precursors for DNA synthesis *via* reduction of ribonucleoside diphosphates by ribonucleotide reductase. The salvage pathway enzymes have recently been the focus of interest in respect to their ability to "activate", by NTP-dependent phosphorylation, a variety of nucleoside analogues with antitumour and antiviral activities [1, 2].

Protein kinases (PK) catalyze the transfer of the γ-phosphate of an NTP donor to as many as 9 different amino-acid residues in proteins, the best characterized being those which phosphorylate the side-chain hydroxyls of Ser and/or Thr (Ser/Thr kinases), or the phenolic hydroxyl of Tyr (protein-tyrosine kinases, PTK). Some of these can phosphorylate all three amino acids, and are known as dual-specificity kinases [3]. Protein phosphorylation is the most prevalent post-translational process in living cells, and protein kinases are key regulators of signal transduction in eukaryotes, from primary transmembrane signalling to control of transcription, translation and cellular metabolism. In contrast to the limited number of known nucleoside kinases, well over 200 PKs have now been identified by purification, molecular cloning and PCR-based methodology; and the rate of increase of this number suggests that as much as 3% of eukaryotic genes may code for such kinases [4].

ATP AS PHOSPHATE DONOR

Kinases are listed by MEDLINE as "Phosphotransferases, ATP", a formulation which is not entirely unambiguous in that there are now numerous examples of kinases which can use other NTPs as effectively as, and frequently more effectively than, ATP, at least *in vitro*, and there is mounting evidence that this is reflected *in vivo*. It is simply a fact that relatively little attention has been directed to correlating the *in vitro* properties of these enzymes with conditions prevailing in intact cells. The view that ATP is the universal *in vivo* donor is based largely on the following considerations:

(a) The high standard free energy change for hydrolysis of the β,γ-phosphoanhydride bond in ATP. But the same holds for any other nucleoside 5'-triphosphate. Furthermore, the free energy change for hydrolysis of ADP to AMP + P_i is virtually the same as that for hydrolysis of ATP to ADP + P_i [5]. On this basis one might anticipate that ADP may be a donor. Furthermore, as recently pointed out by Kornberg [6], inorganic polyphosphate (poly-P_i), with its energy equivalence to ATP, qualifies as a substitute for the latter in its kinase roles, as has in fact been demonstrated experimentally both for poly-P_i and inorganic pyrophosphate (PP_i), discussed in the next section.

(b) ATP usually functions quite well as a phosphate donor in the cell-free systems normally employed to study the properties of kinases. But so do, quite frequently, other NTPs, e.g. protein kinase CK-II (previously known as casein kinase 2) will accept GTP as effectively as ATP. This is, in fact, one of the criteria for distinguishing between CK-II and CK-I.

The classical hexokinase reaction is an example of one with an apparently high degree of specificity for ATP as phosphate donor, at least when the essential chelating divalent cation is Mg^{2+}. Kaji & Colowick [7] initially showed that, amongst all the natural NTPs and dNTPs, only dATP appeared capable of partially replacing ATP. Subsequently Hohnadel & Cooper [8], who measured the kinetic constants of the reaction, reported that both dATP and 3'-deoxy-ATP were poor donors, pointing to a requirement for the ribose *cis*-hydroxyls. Surprisingly, 7-deaza-ATP was as effective a donor as ATP.

Donors other than ATP are not uncommon amongst lipid kinases. Whereas formation of phosphatidate by diacylglycerol kinase exhibits a preference for the ATP donor, dolichol kinase, which catalyzes phosphorylation of endogenous and exogenous dolichols, is CTP-dependent. And membrane fractions from yeast, which catalyze phosphate transfer to membranous lipids, with formation of both phosphatidate and dolichol phosphate, utilize CTP as the sole donor. ATP, GTP and UTP were

inactive as donors, and ATP was not even a detectable inhibitor [9].

(c) The high intracellular concentration of ATP relative to other NTPs. This is obviously highly relevant, and merits consideration from several points of view, not the least of which is the characterization of the kinetics of enzymes for substrates and regulatory ligands relative to their concentrations in the cell. Such information is equally important in pharmacological studies related to development of analogues, which may compete with natural compounds, for chemotherapeutic purposes.

A recent comprehensive survey of literature data on cellular levels of purines and pyrimidines, their nucleosides and nucleotides [10] lists the values shown in Table 1 for the four common NTPs in tissues, and in cultured normal and tumour cells. However, the standard deviations shown in the table do not necessarily reflect the differences in levels listed for various cells, whether mitotic or quiescent; and, as the author points out, it is not unusual for the reported concentration of a given NTP in the same cell line or tissue to vary over a range of 5- to 50-fold, largely as a result of the different experimental techniques employed.

It will be noted, from Table 1, that the NTP levels in normal (resting) cells are lower than in tissues. By contrast, these levels are significantly higher in tumour cells, particularly those of CTP, UTP and GTP relative to ATP. This is further illustrated by the NTP levels in log phase ML-1 human myeloid leukemia cells [11] shown in Table 2, and in MOLT-4 T lymphoblasts [12] listed in Table 3. For the latter, the data include also dNTP levels; it will be seen that these are about 40-fold lower than for NTPs, characteristic of tumour cells in culture.

Table 1
NTP concentrations (mM) in tissues and cultured cells[a]

NTP	Tissues	Normal cells	Tumour cells
ATP	3.5 ± 0.8	2.5 ± 1.2	3.1 ± 2.1
GTP	1.0 ± 0.2	0.23 ± 0.2	0.47 ± 0.2
UTP	0.37 ± 0.08	0.23 ± 0.2	0.7 ± 0.5
CTP	0.09 ± 0.03	0.08 ± 0.13	0.40 ± 0.25

[a]Data from Traut [10].

Table 2
NTP concentrations (mM) in log phase ML-1 human myeloid leukemia cells[a]

ATP	3.5
GTP	0.9
UTP	0.9
CTP	0.4

[a]Data from White & Capizzi [11].

Table 3
Intracellular concentrations of nucleoside triphosphates (NTP) in MOLT-4 T lymphoblasts[a]

NTP concentrations (mM)[b]			
CTP	0.57	dCTP	0.013
UTP	1.51	dTTP	0.055
ATP	3.78	dATP	0.049
GTP	0.87	dGTP	0.022

[a]Data from Shewach *et al.* [12]; [b]Total NTP + dNTP = 6.8 mM.

In normal cells the dNTP levels are 2 to 3 orders of magnitude lower than the corresponding NTPs, and increase 5- to 10-fold in actively dividing cells and tumour cells, although variability in published data is rather high [10]. Data are somewhat sparser as regards compartmentation of NTPs between cytoplasm, nucleus and mitochondria, although it appears that the concentrations of adenine nucleotides, including ATP, are significantly higher in the latter [10].

PHOSPHATE DONORS OTHER THAN NTPs

Before proceeding to a discussion of NTP-dependent kinases, it appears appropriate to at least draw attention to the existence of other phosphate donors in phosphotransferase reactions.

The widespread distribution of poly-P_i in many organisms, and its replacement of ATP as a phosphate donor, e.g. in the phosphorylation of glucose in numerous bacteria, has been the subject of an interesting review by Kornberg [6]. Worthy of note is the fact that these gluco-

kinases utilize either poly-P_i and/or ATP as donors [13], with the phylogenetically ancient species exhibiting a marked preference for poly-P_i.

Inorganic pyrophosphate (PP_i) has also been implicated as the phosphate donor for nucleoside kinases in extracts of some mollicutes, with ATP inactive as donor [14]. PP_i-dependent protein phosphorylation in intact yeast mitochondria has been reported, at a level about 25% that obtained with ATP as donor [15].

A noteable feature of glycolysis is its remarkable conservation throughout evolution, and one of the key enzymes involved is phosphofructokinase, which uses ATP as a donor in a reaction which is irreversible. Recent evidence has revealed the existence, in prokaryotes, higher plants and unicellular eukaryotes of PP_i-dependent phosphofructokinase which, in contrast to the ATP-dependent enzyme, is reversible and may be close to equilibrium *in vivo*. Moreover, the PP_i-dependent enzyme is capable of liquidating PP_i *in vivo* as effectively as inorganic pyrophosphatase, so that the use of PP_i as donor is energetically advantageous. Sequence comparisons of both enzymes from a variety of sources point to their having a common ancestry [16].

During the course of an investigation of the main pathway for fermentation of maltose or cellobiose by the hyperthermophile *Pyrococcus furiosus*, Kengan *et al.* [17] found that the hexokinase and phosphofructokinase activities in cell-free extracts of this organism function only with ADP as donor. Neither PP_i nor ATP were active as donors. This appears to be the first reported instance of an ADP-dependent kinase. It was proposed by the authors that ADP-dependent enzymes may be beneficial in this organism because of its high growth temperature, at which ADP is more stable than ATP. It will be of obvious interest to determine whether other kinases in this organism are equally ADP-dependent, whether other NDPs can replace ADP, and to what extent such ADP-dependent enzymes exist in other thermophiles, and, if so, at what temperature they make their appearance.

In the bacterial phosphotransferase system (PTS), which operates by a mechanism referred to as group translocation, the active transport of sugars into the cells involves their phosphorylation during transport across the cell membrane by means of a system involving several membrane proteins. One unique feature of this system is that phosphoenolpyruvate, and not ATP, is the phosphate donor. It was subsequently found that the PTS also functions as a complex protein kinase system which controls the expression of many genes and regulates a variety of metabolic processes, recently reviewed by Saier & Reizer [18]. It is perhaps of some significance that phosphoenolpyruvate-dependent protein kinase activity, leading to phosphorylation of serine residues in a 25-kDa protein, was earlier reported in the soluble fraction of rat skeletal muscle [19].

A rather unusual mechanism of protein phosphorylation has been described by Seydel & Huttner [20], in which the phosphate donor is 3'-phosphoadenosine-5'-phosphosulfate (PAPS), a ubiquitous nucleotide which is the sulfate donor in cellular sulfation reactions. It was found that PAPS, but not ATP, is the donor for phosphorylation of Ser residues in a wide variety of proteins in rat and bovine tissues, and in mammalian cell lines.

NTP DONORS FOR NUCLEOSIDE KINASES

A distinctive feature of nucleoside kinases is their ability to use a broad range of NTP and dNTP donors, including non-natural synthetic analogues. A typical example is uridine kinase (actually uridine/cytidine kinase) for which, with the exception of UTP and CTP, feedback inhibitors which dissociate the enzyme to monomers, all other NTPs and dNTPs, including even dUTP and dCTP, are good donors [21].

Vertebrate thymidine kinase (TK) exists in two distinct forms, encoded by two separate genes, the cytosolic enzyme (TK1) and the mitochondrial one (TK2). Whereas TK1 can use most NTPs, apart from dTTP and CTP, as donors, TK2 (which also phosphorylates deoxycytidine, and hence is really a pyrimidine deoxynucleoside kinase), effectively utilizes both ATP and CTP [22].

Particularly interesting are viral thymidine kinases, considered to have evolved from cellular deoxycytidine kinase [23]. Many of these will phosphorylate a more diverse range of nucleoside analogues than their cellular counterparts; and this is indeed the basis for development of nucleoside analogues as anti-

viral drugs, the best-known example of which is the antiherpetic agent acyclovir [2, 23]. Most of these viral TKs will accept NTP donors other than ATP [2, 23, 24], one apparent exception being vaccinia virus TK, for which GTP, UTP, CTP are very poor donors, if at all [25].

Adenosine kinase from chick liver was long ago shown to effectively utilize a broad range of NTPs as donors, with GTP almost twice as effective as ATP [26]. On the other hand, it has recently been shown that anoxic rat hepatocytes contain an activity which co-purifies with adenosine kinase, and catalyzes an exchange reaction between AMP and adenosine [27], so that phosphorylation of the latter is not necessarily due to the kinase activity.

The foregoing exchange reaction is a well-known one under normal physiological conditions, catalyzed by so-called nucleoside phosphotransferases, such as cytosolic 5′-nucleotidases (and even non-specific phosphatases), and are frequently involved in the activation, by phosphorylation, of some chemotherapeutic nucleoside analogues [2, 22]. We now proceed to a more detailed survey of the NTP donor properties of mitochondrial deoxyguanosine kinase (dGK) and deoxycytidine kinase (dCK).

2′-Deoxyguanosine kinase (dGK)

This is a striking example of a nucleoside kinase with broad donor specificity. The enzyme has been isolated from mitochondria and purified to near homogeneity, and the only natural acceptors are dGuo and dIno [28]. Since the pH optimum was found to have the unusual low value of 5.5, the authors examined the donor properties of various NTPs and dNTPs over a range of pH values, with results listed in Table 4.

It will be noted that, at the pH optimum of 5.5, ATP is indeed the best donor, while UTP is 50% as effective. As the pH is raised, the activity of ATP markedly decreases. Concomitantly those of UTP, dTTP, CTP, dCTP increase dramatically. At physiological pH, UTP and dTTP are twice as effective as ATP, while CTP and dCTP are equally as effective.

At pH 7.5 the K_m values for ATP, UTP and dTTP were 780, 125 and 80 μM, respectively. Hence, with the 10 mM NTP concentrations employed as donors, enzyme saturation would be expected to vary only from 93% to 99%, whereas variations in the reaction rate are more than 2-fold. It obviously would have been useful to measure the K_m parameters at the other pH values.

Table 4

Relative pH-dependent donor activities of various Mg^{2+}-nucleoside triphosphates (10 mM) for phosphorylation of dGuo (10 μM) by highly purified dGK from bovine mitochondria [a]

Phosphate donor	pH			
	5.5	6.5	7.5	8.5
ATP	100 (0.214)[b]	100 (0.048)[b]	100 (0.039)[b]	100 (0.031)[b]
dATP	3	10	14	18
UTP	52	157	193	203
dTTP	28	122	232	237
CTP	31	125	125	126
dCTP	27	78	97	115
GTP	10	24	21	18
dGTP	0	0	0	0
dITP	0	0	0	0

[a]Data from Park & Ives [28]; [b]Absolute activities, with ATP as donor, in nmol/min per ml.

The enzyme has since been purified from other, including human, sources [22], and has been reported capable of phosphorylating several synthetic chemotherapeutically active nucleoside analogues. But all such studies were limited to the use of only ATP as donor, so that their relevance to the situation prevailing in intact cells is limited.

2′-Deoxycytidine kinase (dCK)

This enzyme, which also phosphorylates dAdo and dGuo, has been more extensively investigated than any other nucleoside kinase [29], in part because of its ability to activate, by phosphorylation, a variety of nucleoside analogues with antitumour and antiviral activities, several of which have moved into the clinic or clinical trials. These include araC, the most effective drug for treatment of acute myeloid leukemia, the antitumour drug 2-chlorodeoxyadenosine, etc. [2, 11, 22].

Although long known that UTP is a better *in vitro* donor than ATP for this enzyme, most studies of its properties have been based on the exclusive use of ATP as phosphate donor. At-

tention was first directed to the potential intracellular role of UTP by White & Capizzi [11], based on the finding that the intracellular concentration of araC for half-maximal phosphorylation by leukemic blasts from patients was about 2 μM, hence an order of magnitude lower than the *in vitro* K_m of dCK for araC with ATP as donor. Further *in vitro* studies, using as donor a mixture of NTPs corresponding to their concentrations in the same cells (Table 2), coupled with sequential deletion of each NTP from the donor mixture, provided compelling evidence for earlier proposals that UTP may be the important intracellular phosphate donor for dCK.

Additional supporting evidence for the foregoing was adduced by Shewach *et al.* [12], using purified dCK from cultured Molt-4 T lymphoblasts. Following determination of the NTP and dNTP levels in these same cells (see Table 3), it was first shown that GTP, dGTP and dTTP were as effective as, and UTP markedly superior to, ATP as phosphate donors, with dCyd as acceptor.

Kinetics for dCyd phosphorylation *in vitro* were then conducted, using as donors ATP alone, UTP alone, and an NTP mixture corresponding to the intracellular concentrations of the NTPs and dNTPs (Table 3), with results exhibited in Table 5. It will be seen that UTP alone at 1.5 mM is much more effective than ATP at 2 mM, with K_m values of 0.23 μM and 0.8 μM, and V_{max}/K_m = 7.9 and 4.9, respectively. Quite striking was the efficacity of the NTP mixture (K_m = 0.35 μM, V_{max}/K_m = 11) and the dramatic 7-fold drop in donor efficiency on removal of UTP from the NTP mixture (K_m = 1.5 μM, V_{max}/K_m = 1.6). In part this is due to liquidation by UTP of the known feedback inhibition by the distal end product of dCyd phosphorylation, viz. dCTP.

Recalling once again the key role of dCK in activation of chemotherapeutic nucleoside analogues, it is to be anticipated that the foregoing results will stimulate further such studies, preferably with the aid of NTP mixtures containing at least one [γ-^{32}P]NTP in order to unequivocally identify the source of the phosphate on the acceptor employed [30].

The results of an initial study of this nature have now been reported [31], using highly purified human leukemic cell dCK, and with both dCyd and dAdo as acceptors. It was first

Table 5
Kinetics for dCyd phosphorylation by MOLT-4 deoxycytidine kinase [a]

Phosphate donor	K_m (μM)	V_{max}/K_m
2.0 mM ATP	0.8	4.9
6.8 mM ATP	1.9	5.1
1.5 mM UTP	0.23	7.9
6.8 mM NTP[b]	0.35	11.0
6.8 mM NTP – 1.5 mM UTP	1.54	1.6

[a]Data from Shewach *et al.* [12]; [b]Total NTP + dNTP in MOLT-4 cells (see Table 3).

shown that, as with the MOLT-4 enzyme, UTP alone was 60% more effective than ATP with dCyd as acceptor. UTP alone was also more effective with dAdo as acceptor, but to a smaller extent, and probably reflecting interdependence between donor and acceptor specificities. Using equimolar mixtures (50 μM:50 μM and 1 mM:1 mM) of phosphate-labelled ATP and cold UTP, and low and high concentrations of dCyd and dAdo as acceptors, phosphate transfer from ATP, relative to total phosphate transfer by the ATP:UTP donor mixture, ranged from only 3% to 10% (see Table 6). With an ATP/UTP ratio of 6:1, hence exceeding the intracellular ratio of these two components, especially in tumour cells, phosphate transfer from ATP to both acceptors did indeed increase, but still did not exceed 40% (Table 6).

It is clearly desirable to extend this approach to other nucleoside kinases. The availability of highly purified cytoplasmic thymidine kinase (TK1) and mitochondrial thymidine kinase (TK2) from human leukemic spleen was profited from to carry out several preliminary experiments, with dThd as acceptor. UTP alone proved to be a moderate donor with TK2, and somewhat poorer with TK1, relative to ATP. But, with an equimolar mixture of phosphate-labelled ATP and cold UTP (50 μM each) as donor, and 1 μM Thd as acceptor, TK2 gave no detectable phosphate transfer from UTP to Thd. With the TK1 enzyme, using 15 μM Thd as acceptor, phosphate transfer to Thd from UTP attained a level of only 12% of the total phosphate transferred. Again, with TK2 and dCyd as acceptor, and an ATP:CTP (50 μM:50 μM) mixture as donor, phosphate transfer from

Table 6
Deoxycytidine kinase-catalyzed relative transfer of phosphate to dCyd and dAdo from the [α-^{32}P]ATP component of an ATP/UTP donor mixture[a]

Phosphate acceptors		Phosphate donors, ATP : UTP		
		50 μM:50 μM	1 mM:1 mM	300 μM:50 μM
(μM)		% phosphate uptake from ATP		
dCyd	2.5	5	4	29
	100	10	8	41
dAdo	100	3	8	34
	1000	3	8	23

[a]Data from Krawiec *et al.* [31].

CTP was equal to that from ATP. These findings are presently being extended, including the use of other potential NTP donors (Krawiec, K., Kierdaszuk, B. & Shugar, D., unpublished).

Extension of the foregoing approach to other nucleoside kinases is clearly called for. Particularly interesting would be the behaviour of dGK in light of the results shown in Table 4, above.

THE NTP DONOR FOR PROTEIN KINASES

Notwithstanding several well-known exceptions, it is still widely and implicitly assumed that ATP must be the intracellular phosphate donor for protein kinases, and relatively little effort has been devoted to possible involvement of other NTPs.

The best-known exception is protein kinase CK-II, which accepts GTP as effectively as ATP, both for autophosphorylation and phosphorylation of protein substrates, one of the criteria for distinguishing CK-II from CK-I. By contrast, rat liver p75 PTK requires ATP and is inactive with GTP ([32], see also below).

The most striking example, just recently reported [33], is PKC, earlier claimed by several observers to be inactive with GTP. However, the mammalian PKC family comprises at least 12 different related polypeptides (isotypes), denoted by Greek symbols. Studies in a variety of cell types have underlined the different biological effects of these isotypes, most likely due, at least in part, to individual intrinsic differences in substrate specificities, reviewed by Dekker & Parker [34]. This was subsequently admirably corroborated by Kielbassa *et al.* [35], who demonstrated that the elongation factor eEF-1α is selectively phosphorylated by PKCδ, and that a peptide with a sequence corresponding to that of residues 422–443 from murine eEF-1α, and containing Thr-431 of the latter, is an absolutely specific substrate for PKCδ. The authors did not, however, envisage a change in specificity for the NTP donor, which is also a substrate. It has now been shown that one of these isotypes, PKCδ from porcine spleen, which was apparently unable to phosphorylate various substrates in the presence of GTP, undergoes autophosphorylation with GTP six times more effectively than with ATP [33]. Furthermore, mouse brain cPKC (a mixture of the α, β and γ isotypes) was also found capable of utilizing GTP, but only 25% as effectively as ATP. Overall, then, PKCδ is autophosphorylated 24-fold more effectively with GTP than cPKC. The autophosphorylation reaction was found to be intramolecular, and the catalytic subunit of PKA, as well as of PTK_{src}, tested for comparison purposes, were only minimally, if at all, autophosphorylated by GTP.

Even more striking was the observation that, while both Ser and Thr residues were autophosphorylated with either ATP or GTP, the level of such phosphorylation was much higher with GTP. Hence some sites phosphorylated with GTP must differ from those phosphorylated with ATP, as was indeed demonstrated by phosphopeptide mapping [33]. It remains to be shown to what extent such autophosphoryla-

tion, with either ATP or GTP, affects the activity and specificity of the isotypes towards protein substrates.

Role of divalent cations

NTP-dependent protein kinases require a chelating divalent cation, usually Mg^{2+}, which bridges oxygens of the β- and γ- phosphates of the donor. Ser/Thr kinases can frequently utilize Mn^{2+} less effectively than Mg^{2+}, whereas the reverse holds for PTKs. The enzymes may bind more than one cation, e.g. the catalytic subunit of phosphorylase kinase contains two such binding sites, and binding of Mn^{2+} in place of Mg^{2+} at the second site has been shown to inhibit phosphorylation of the seryl residues of phosphorylase *b* [36]. Furthermore, the acceptor specificity of the catalytic subunit, as well as of the holoenzyme, of phosphorylase kinase is dependent on the nature of the second bound cation. With Mg^{2+}, seryl phosphorylation of phosphorylase *b* occurs, whereas Mn^{2+} leads to tyrosyl phosphorylation of angiotensin II. This is, in fact, an example of a so-called dual-specificity PK [3], in this case dependent on the NTP chelating agent. The ability of some PKs to discriminate between Ser/Thr and Tyr has been reviewed in remarkable detail by Taylor *et al.* [37], based on a comparison of the crystal structures of the kinase domain of the insulin receptor PTK with that for PKA, but without reference to the essential role of the nature of the divalent cation, as revealed by enzymatic studies such as that of Yuan *et al.* [36], above. The role of the divalent cation has been nicely depicted in the crystal structure of the ternary complex of porcine heart PKA complexed with an inhibitory peptide and the non-hydrolyzable bidentate Mn^{2+}-adenylyl imidophosphate, bearing in mind that PKA is still active with Mn^{2+} [38].

An additional example of the role of divalent cations is the reported existence in sheep platelets of two PTKs, one of which required Zn^{2+} and was inactive with Mg^{2+}, while the other required Mg^{2+} and was inactive with Zn^{2+}. The two activities were also distinguished by their differential responses to inhibitors [39]. Stimulation of some PTKs by Zn^{2+} had been previously reported by others.

In the foregoing study, and that of Yuan *et al.* [36], only ATP was used as donor. But there are instances where the nature of the divalent cation dictates donor specificity.

Rat liver p75 PTK, purified to near homogeneity, exhibited a strict requirement for Mn^{2+} with ATP as donor, whereas with Mg^{2+} and other cations, activity was less than 2% that with Mn^{2+}. By contrast, pp60 and EGF-R kinases utilized GTP with 40% the efficiency of ATP [32]. Insulin-mediated phosphorylation of both the α- and β- subunits of its own receptor required Mn^{2+} or Co^{2+} with ATP. Both Mg^{2+} and Zn^{2+} were ineffective, and GTP could not replace ATP, in contrast to the reported EGF-mediated phosphorylation of EGF-receptors, where GTP readily replaced ATP [40].

A Ser/Thr PK activity, isolated from HeLa nuclei, resembled in some respects kinases CK-I and CK-II from the same nuclei. The Mg^{2+} requirement could be replaced by Mn^{2+}. With Mg^{2+} the donor was ATP; but with Mn^{2+} both ATP and GTP were good donors [41]. The activity was, however, purified only 50- to 100-fold, and its characterization was not unequivocal.

NTP inhibitors of protein kinases

Numerous NTP analogues have been synthesized and examined as potential inhibitors of protein kinases, in part with a view to mapping binding sites, and Table 7 lists the K_i values for some of these *vs* protein kinases CK-I, CK-II and PKA. Such data were collected largely in the 1980's, prior to the advent of PK crystal structures ([30, 42] and references cited). They are still deserving of some attention if one bears in mind that, notwithstanding the enormous information forthcoming from crystal structures of macromolecules, and their complexes with ligands, the resulting picture is a static one, the interpretation of which requires kinetic, biochemical and genetic data, and NMR spectroscopy where feasible, and well exemplified by its application to phosphoglycerate kinase [43]. This situation is in process of being dramatically improved by application of Laue diffraction with the polychromatic high-intensity radiation from a synchrotron source, to obtain time-resolved patterns of the course of an enzymatic reaction; and has already been successfully applied to identify the structures of two sequential intermediates formed during the catalytic reaction of isocitrate dehydrogenase [44].

Table 7

Representative data for inhibition of several protein kinases by NTP analogues, all competitive vs ATP [a]

NTP	K_i (μM)		
	Kinase CK-I	Kinase CK-II	PKA
N^6-methyl-ATP	120	165	180
N^6-dimethyl-ATP	340	42	2600
N^6-benzyl-ATP	7	17	20000
N(1)-methyl-ATP	–	40	330
GTP	3000	9	2100
ITP	480	25	13000
UTP	540	182	–
CTP	160	315	–
Purine riboside-TP	1250	113	1050
7-deaza-ATP	–	72	9
8-amino-ATP	45	135	33
8-bromo-ATP	110	100	150
8-azido-ATP	340	175	120
2′-deoxy-ATP	74	21	30
3′-deoxy-ATP	9	4	1.5
3′-amino-3′-deoxy-ATP	6	11	10
ATPγS	17	18	22

[a]Data from Flockhart *et al.* ([42] and references cited). See also Shugar [30] for further details and references.

Reverting to Table 7, the NTP analogues listed, all competitive with respect to ATP, were tested only as potential inhibitors, with no regard to the possibility that a given NTP may "inhibit" by virtue of its being an alternative substrate. The low K_i for GTP with CK-II, but not CK-I and PKA, is consistent with its known ability to replace ATP as a donor for the former. The K_i for ITP, a close analogue of GTP, is equally low (25 μM), and may well prove to be a donor for CK-II. Note that *N*(1)-methyl-ATP (K_i 40 μM) is in the fixed imino form, hence structurally analogous to ITP, although it is protonated at physiological pH (pK = 8.7).

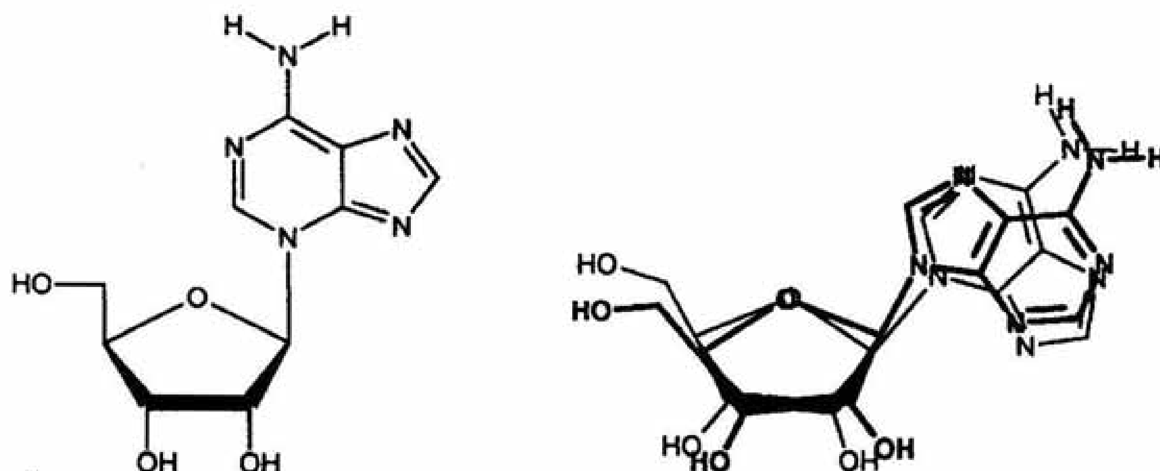
Quite striking are the low K_i values of 3′-dATP and 3′-amino-3′-dATP for all three enzymes. Both 2′-dATP and 3′-dATP have been shown to be weak donors, and 3′-amino-3′-dATP almost as effective as ATP, for phosphorylation of the sarcoplasmic reticulum Ca^{2+}-ATPase [45].

8-Azido-ATP, commonly employed as a photoaffinity ligand, can replace ATP in phosphorylation of the P protein of vesicular stomatitis virus by its L-protein associated kinase activity ([30, 46] and references cited).

The low K_i values of N^6-benzyl-ATP for CK-I and CK-II (7 μM and 17 μM), as compared to 20 mM for PKA, underline, contrary to widespread belief, the ability of an ATP-competitive inhibitor to exhibit selectivity *vs* a given kinase or class of kinases, a subject discussed in more detail elsewhere [46].

The low K_i of 7-deaza-ATP with PKA (9 μM) may be relevant to the report (see above) that this analogue is as effective a donor as ATP in the hexokinase reaction [8].

The fact that 8-bromo-ATP is only a moderate inhibitor of all three enzymes was attributed to its being in the *syn* conformation about the glycosidic bond, based on the earlier observation that 8-bromopurine nucleosides exhibit this conformation in the crystal. But it has elsewhere been shown that, in solution, these nucleosides exhibit only a preference for the *syn* conformation, and may be constrained to the *anti* conformation on binding to an enzyme [47].



Scheme 1. 3-(β-D-ribofuranosyl)adenine (3-β-Ado), shown (left) in the syn conformation about the glycosidic bond, and superimposability of 3-β-Ado in the syn conformation on adenosine in the anti conformation.

Relevant to the above is 3-(β-D-ribofuranosyl)adenine (3-β-Ado, Scheme 1), the triphosphate of which (3-β-ATP) was originally shown by Leonard & Laursen [48] to replace ATP in the hexokinase phosphorylation of glucose, in the myokinase system, and in production of light by the luciferin-luciferase system, while 3-β-ADP could substitute for the ADP component of $NADP^+$. Subsequently Hohnadel & Cooper [8] showed that the kinetic constants for ATP and 3-β-ATP in the yeast hexokinase reaction were comparable. With the purine nucleoside phosphorylases from *E. coli* and a mammalian source (calf spleen), we have found that 3-β-Ado and 3-β-Ino can substitute for Ado and Ino to varying extents as substrates (Bzowska, A., Kulikowska, E. & Shugar, D., in preparation). All of these results are readily interpretable by the proposal of Leonard *et al.* [49] that 3-β-Ado in the *syn* conformation about the glycosidic bond is superimposable on ATP in the *anti* conformation, as shown in Scheme 1. And, in fact, 3-β-Ado in the crystal is in the amino form, as shown in Scheme 1, and in the syn conformation [50]. In solution it is known to be in the amino form, and most likely exhibits a *syn-anti* equilibrium in favour of the *syn* conformation. The foregoing therefore does not permit one to conclude that it is the *syn* conformation of 3-β-Ado, or its di- and triphosphates, which replace Ado, ADP and ATP in the enzymatic reactions, since ATP in the *syn* conformation is equally superimposable on 3-β-ATP in the *anti* conformation.

Worthy of note is ATPγS, with K_i in the range 17–22 μM for "inhibition" of all three enzymes (Table 7). It was only subsequently found that ATPγS is a donor for protein kinases, enabling the preparation of thiophosphorylated proteins and synthetic peptides which are relatively resistant to protein phosphatases [51]. Actually NTPγS analogues are also relatively resistant to non-specific phosphatases in general, and this has been profited from to employ GTPγS (which is resistant to GTPases) in place of GTP to study the crystal structure of the complex of the activated α-subunit of a heterotrimeric G protein, viz. the complex of transducin-α with GTPγS [52].

Since there are a number of protein kinases which accept ATPγS as a donor, it obviously would be of interest to examine GTPγS as a potential donor for those kinases which utilize GTP, in some cases as effectively as ATP, e.g. protein kinase CK-II. Srivastava *et al.* [53] have reported that GTPγS inhibits phosphorylation of the insulin receptor and a novel GTP-binding protein from human placenta. GTPγS was used in place of GTP because the GTP-binding protein, G_{ir}, possesses GTPase activity; but the possibility was apparently not envisaged that, like ATPγS, GTPγS might be a donor.

No consideration appears to have been given to the donor and/or inhibitor properties of NTPγS analogues in other kinase systems. We have found that, with human cytosolic thymidine kinase and deoxycytidine kinase, ATPγS is not a donor, but is a feeble inhibitor

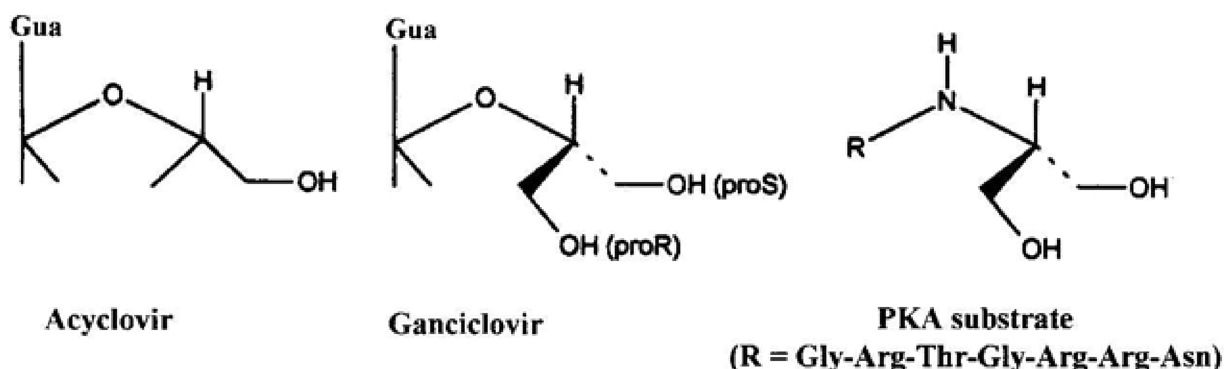
(Krawiec, K., Kierdaszuk, B. & Shugar, D., unpublished).

PROTEIN KINASES WITH NON-PEPTIDE SUBSTRATES

Over the years there have been sundry reports of protein kinases with the ability to phosphorylate low-molecular mass non-peptide substrates, e.g. three laboratories have described the association of glycerol kinase activity with pp60^{src}, the Rous sarcoma virus transforming gene product, or its variants [54–56]. Particularly interesting was the finding of Graziani *et al.* [55] that, whereas GTP serves as a phosphate donor for autophosphorylation of the enzyme, and for its phosphorylation of α-casein, it could not replace ATP in the glycerol phosphorylating activity of pp60^{src}. In the study of Richert *et al.* [54], GTP was not tested as a possible donor, but both ATP and dATP were equally effective for phosphorylation of casein and glycerol. Variants of pp60^{src} were also found to phosphorylate at significant levels tyramine and *N*-acetyl tyrosine, but not tyrosine [56].

The ability of pp60$^{v\text{-}src}$ to phosphorylate glycerol, and a report that phosphatidylinositol turnover is stimulated in RSV-transformed cells, prompted Sugimoto *et al.* [57] to examine relevant lipids as potential substrates of pp60^{src}. It was, in fact, found that the purified enzyme phosphorylated phosphatidylinositol to its mono- and diphosphorylated derivatives, as well as diacylglycerol to give phosphatidic acid. These activities exhibited the same thermolability, and sensitivity to inhibitors as the casein kinase activity of pp60^{src}. Independently Macara *et al.* [58] demonstrated that p68$^{v\text{-}ros}$, the transforming protein of avian sarcoma virus UR2, possesses associated phosphatidyl kinase activity, and that turnover of phosphoinositides *in vivo* was significantly enhanced by transformation of chicken cells with UR2. Subsequently it was shown by Georgoussi & Heilmeyer [59] that phosphatidyl kinase, but not diacylglycerol kinase, activity is associated with phosphorylase kinase, and that a monoclonal antibody directed specifically *vs* the α-subunit of the latter coprecipitated both activities. In all the foregoing only ATP was employed as phosphate donor. Bearing in mind the known CTP-dependence of some lipid kinases, referred to above, it would have been of interest to examine activities with CTP. It would have been equally useful to run trials with GTP, which can often replace ATP for protein-tyrosine kinase activities, but is probably not a donor for lipid kinases.

The antiviral activities of acyclonucleosides, such as acyclovir, are due to their selective phosphorylation by viral, and not host cell, thymidine kinases. A close structural analogue of acyclovir, ganciclovir (Scheme 2), is a broad-spectrum agent *vs* DNA viruses, including human cytomegalovirus (HCMV), following its phosphorylation in HCMV-infected cells. But HCMV does not code for a TK. It was then found that phosphorylation of ganciclovir in the presence of ATP is due to the product of the HCMV *UL97* gene, which turns out to be a protein kinase homologue [60, 61], and mutations in this gene confer resistance to ganciclovir [62]. No protein substrate has yet been



Scheme 2. The antiviral agents acyclovir and DHPG, and a synthetic peptide substrate for cAMP-dependent protein kinase (PKA).

Note the similar prochiral hydroxyls in DHPG and the PKA substrate. The pro-(*S*) hydroxyl of DHPG is phosphorylated by the *UL97* gene product of human cytomegalovirus, and the corresponding hydroxyl of the peptide by PKA. See text for further details.

found for the *UL97* gene product. The ability of a PK homologue to phosphorylate a monomeric non-peptide acyclonucleoside analogue is tantalizing. Attention has also been drawn to the fact that ganciclovir possesses prochiral hydroxyls (see Scheme 2) and that probably only one of these is phosphorylated stereospecifically by the *UL97* gene product, in a manner strikingly reminiscent of the stereospecific phosphorylation by cAMP-dependent PK (PKA) of a synthetic peptide substrate with similar prochiral hydroxyls [30, 63, 64].

It was subsequently shown by Metzger *et al.* [65] that recombinant vaccinia virus containing the HCMV *UL97* gene exhibits susceptibility to ganciclovir, as anticipated. The same authors reported that phosphorylation of ganciclovir, in cells infected with the recombinant virus, was totally inhibited by addition of 100 μM guanosine, and concluded that guanosine is also a substrate for the *UL97* gene product. However, this proposal may be premature in that, although a guanosine kinase has been isolated from a protozoan parasite, *Trichomonas vaginilis*, no such activity has hitherto been identified in a mammalian source [2].

It is clear that one must await isolation and purification of the *UL97* gene product. Meanwhile, one possible approach to further clarification might be an examination of the donor specificity for phosphorylation of ganciclovir. As pointed out above, viral TKs are capable of utilizing a variety of NTPs as phosphate donors. By contrast, protein kinases are somewhat more stringent in their requirement for an NTP donor (see above). It may, consequently, be useful to determine the NTP donor requirements of the *UL97* gene product for phosphorylation of ganciclovir and, possibly, acyclovir, which Metzger *et al.* [65] report to be a weaker substrate, although no details are given.

Another example of a protein kinase which is capable of phosphorylating a monomeric non-peptide acceptor is hexokinase 1 (HK1) from rat brain. This undergoes autophosphorylation at Ser, Thr and Tyr, and is therefore a so-called dual-specificity protein kinase. But the enzyme also phosphorylates histone H2A, although phosphorylated sites were not determined. Protein kinase activity was observed only in the absence of glucose, and was completely suppressed on addition of 0.2 mM glucose [66]. Hexokinase autophosphorylation has been reported by others [67]. At least in the case of the hexokinase isozyme PII of *Saccharomyces cerevisiae*, which undergoes intramolecular autophosphorylation and will also phosphorylate histone III-S and α- or β-casein, it was shown that protein kinase activity resides in a different domain of the protein from the hexose-phosphorylating activity [68]. In all the foregoing, only ATP was used as the phosphate donor. Recalling the more stringent requirement of yeast hexokinase for ATP, and the ability of some protein kinases to utilize GTP, it would be of interest to examine the donor requirements of the two activities exhibited by these hexokinases.

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