Iontophoresis of Bases, Nucleosides, and Nucleotides

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Purpose. To investigate whether transdermal iontophoresis may be potentially useful for delivery of oligonucleotide drugs, the electrotransport of representative bases (uracil and adenine), nucleosides (uridine and adenosine) and nucleotides (AMP, ATP, GTP and imido-GTP) across mammalian skin *in vitro* has been considered.

Methods. While the passive permeability of all compounds investigated (from 1 mM solutions at pH 7.4) was very low, the application of constant current iontophoresis (0.55 mA/cm^2) significantly enhanced the transport of both charged and uncharged species.

Results. The efficiency of delivery depended only weakly upon lipophilicity, varied quite linearly with concentration (for AMP and ATP), was inversely sensitive to molecular weight, and was strongly influenced by charge. Neutral solutes were delivered better from the anode than the cathode, as expected; post-iontophoresis, passive permeabilities were greater than those of the untreated controls, suggesting that iontophoretically-induced changes in barrier function cannot be completely repaired in *in vitro* model systems. The triphosphate nucleotides, ATP and GTP, were essentially completely metabolized (presumably to their corresponding mono-phosphates) during their iontophoretic delivery, while imido-GTP was apparently resistant to enzymatic attack; however, comparison of the transport data from AMP and ATP suggested that ATP metabolism occurred primarily *after* the rate-limiting step of iontophoresis.

Conclusions. The results obtained are consistent with the general patterns of behavior previously observed in investigations of amino acid and peptide electrotransport. It remains to be seen whether extension of the research described here to larger oligonucleotide species is a feasible long-term objective.

KEY WORDS: iontophoresis; electroosmosis; transdermal delivery; skin penetration; bases; nucleosides; nucleotides.

INTRODUCTION

Oligonucleotides represent a relatively new class of therapeutic agents, which have the potential to exert a powerful influence on biotechnology through their ability to modify genetic expression (and, hence, to have a significant impact upon the treatment of cancer and viral diseases) (1, 2). However, for oligonucleotides to realize their potential, several severe criteria must be satisfied (3), not the least of which is the question of delivery to the site of action (4). The latter challenge is particularly related to their large molecular weights (~3 Kdaltons and higher), their relative instability (i.e., short biological half-lives), and (at physiological pH) their negative charge.

Recently, iontophoresis (5)—the enhanced transdermal delivery of drugs by an applied electrical field—has been examined for its ability to provide a non-parenteral route of administration for the newer molecules emerging from the biotechnology industry (e.g., peptides and small proteins) (6). While some success has been achieved with relatively small polypeptides, the "structure-activity" relationships governing the iontophoretic delivery of these species have not been fully characterized. Self-evidently, there are a number of variables, which must be examined carefully, including size, charge, lipophilicity, conformation and solubility. With the need for more fundamental information in mind, therefore, we previously examined the iontophoretic delivery of several amino acids, and of a series of tripeptides, as the first step towards characterizing the "structure-activity" relationship(s) for the electrotransport of proteins (7, 8). Following a parallel strategy, we present here a study in which we have determined the efficiency of iontophoresis as an approach to deliver the "building blocks" of oligonucleotides (specifically, various bases, nucleosides and nucleotides).

MATERIALS AND METHODS

The design of the experiments, and their execution, followed closely that described in the earlier studies of Green *et al.* (7, 8).

Chemicals

Tritiated [5,6-^{3}H]uracil, [2,8-^{3}H]adenine, [^{3}H(G)]adenosine monophosphate (AMP) and adenosine triphosphate (ATP) were purchased from NEN (Wilmington, DE). Tritiated [5,6-^{3}H]uridine and [2,8-^{3}H]adenosine came from Moravek Biochemicals (Brea, CA). Tritiated [8-^{3}H]guanosine triphosphate (GTP) and imido[8-^{3}H]guanosine triphosphate (imido-GTP) were obtained from Amersham (Arlington Heights, IL). Radiochemical purity (≥97%) was checked by thin layer chromatography. The corresponding unlabeled chemicals were acquired from Sigma Chemical Company (St. Louis, MO) and Aldrich Chemical Company (Milwaukee, WI). Relevant physicochemical data for the compounds studied are given in the Table.

Apparatus

In vitro flux measurements were made in flow-through diffusion cells (Laboratory Glass Apparatus, Berkeley, CA), which have been previously described [7, 8]. The skin membrane used was either (a) full-thickness tissue from hairless mice (HRS/hr hr, 8–10 weeks old from Simonsen Laboratories, Gilroy, CA), removed at sacrifice and used immediately, or (b) fresh human skin (from abdominoplasty), dermatomed to 600 μm, and used within 24 hours of excision. Current was delivered from custom-made (7) silver–silver chloride electrodes (materials from Aldrich Chemical Co., >99.99% pure). The custom-built power supply (Professional Design and Development Services, Berkeley, CA) was interfaced to a Macintosh IIfx computer (Apple Computer Inc., Cupertino, CA) running Labview software (National Instruments Inc., Austin, TX).

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Table 1. Pertinent Physicochemical Data of the Compounds Studied

Chemical	Molecular Weight (da)	log P[a]	pK_a values[b]
Uracil	112.1	−1.03	9.3
Adenine	135.1	−0.16	4.1, 9.7
Uridine	244.2	−1.98	9.3, 12.6
Adenosine	267.2	−1.10	3.4
AMP	347.2	−2.47	3.8, 6.2
ATP	507.2	−2.62	4.1, 6.5
GTP	523.2	−3.41	3.3, 6.5, 9.3
Imido-GTP	522.2	−3.63[c]	3.3, 7.7[d], 9.3

[a]P = octanol/pH 7.4 aqueous buffer partition coefficient (MedChem Project, Pomona College, Claremont, CA).

[b]Values obtained from the literature (L. Stryer, *Biochemistry* (3rd Edition), W.H. Freeman, New York, 1988).

[c]Estimated using PCModels, version 4.2, (Daylight Chemical Information Systems, Irvine, CA).

[d]It is assumed that the pK of the terminal phosphate group is identical to that of imido-ADP [9].

Procedures

Cathodal and anodal delivery of the bases and nucleosides were evaluated; the nucleotides, on the other hand, were iontophoresed only from the cathode. Parallel passive diffusion experiments were also performed in the absence of current. All solutions were freshly prepared prior to a transport measurement; the permeant concentration was usually 1 mM, the solution having been 'spiked' with radioactivity at 1 μCi/mL. Higher molar concentrations of AMP and ATP were also studied. The background electrolyte, which was used to fill both the 'non-working' electrode compartment and the receptor phase, and in which the permeant was dissolved, was pH 7.4 HEPES (25 mM)—buffered saline (250 mM). The electrode chambers contained 1 mL of solution; the receptor phase, which was degassed under vacuum prior to usage, was perfused at 3–4 mL/hr, with samples being collected automatically every 1.5 hr. Constant current was delivered to the electrodes at 0.55 mA/cm^2 for 12 hours; transport was monitored during this period and for the next 8–12 hours. It was found that all compounds tested, *except* adenine, were electrochemically stable. Adenine, however, as shown by cyclic voltammetry, reacted at both the anode and cathode (and significantly adsorbed to the electrodes passively, without current passage). It was therefore necessary, in the adenine experiments, to connect the donor compartment of the diffusion cell to the electrode chamber via a salt bridge (3% w/v agarose containing 1M NaCl).

Assay Methods/Data Analysis

Permeation of all compounds studied was first assessed simply using liquid scintillation counting of the collected receptor phase fractions. The radioactivity in each sample was mathematically corrected for the amount of permeant which had crossed the skin during the sampling period but which had not yet reached the sampling vial (due to the finite flow-rate and volume of receptor fluid). The counts were then converted to flux ($nmol/cm^2/hr$). For ATP, GTP and imido-GTP, however, electrochemical degradation in the donor solution, and enzymatic biotransformation in the skin and/or receptor phase, was estimated using the luciferase-luciferin assay. Luciferase catalyzes the oxidation of luciferin in the presence of ATP, Mg^{2+} and oxygen to generate oxyluciferin and light, which is then detected in a bioluminometer (Monolight 2001) [10]. For the assay, 400 μL samples of the receptor solution (or donor elec-

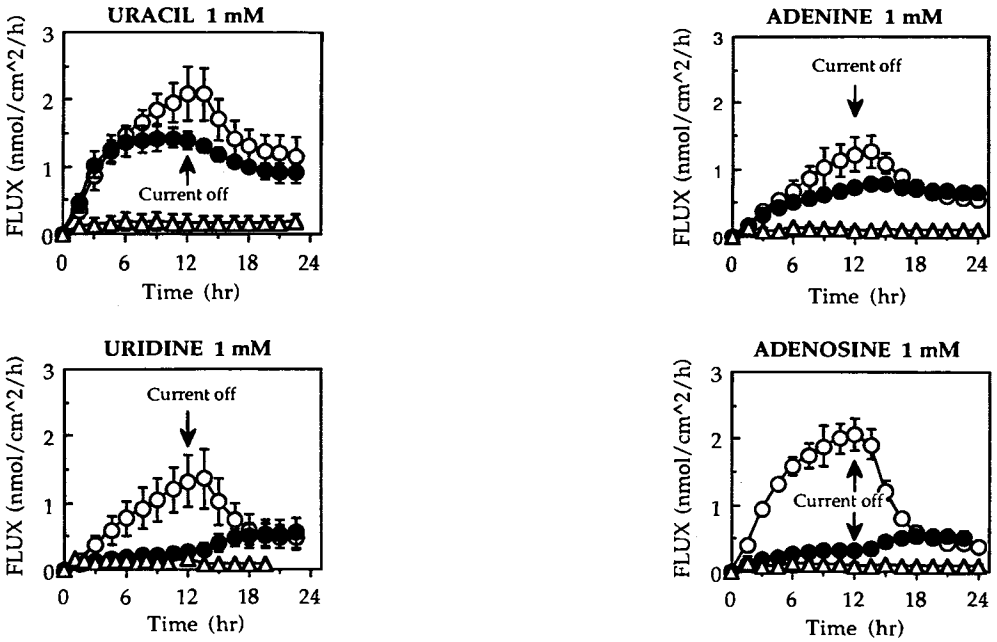

Fig. 1. Fluxes of uracil, adenine, uridine and adenosine across hairless mouse skin *in vitro*, as a function of time, following either iontophoretic (12 hours of constant current [0.55 mA/cm^2]: anodal, open circles; cathodal, closed circles) or passive (open triangles) delivery. Each data point represents the mean ± SD of 3–9 measurements.

trode phase) from the ATP experiments were mixed with 100 μL of luciferase-luciferin solution (4 mg/mL), and the emitted light was measured. A previously constructed standard curve was then used to deduce the quantity of intact ATP in the sample. To assay GTP, the sample was first reacted with ADP in the presence of nucleoside diphosphate kinase, thereby transferring a phosphate and forming ATP [11], which was then measured as above. The transport of the compounds studied, expressed as the flux (nmol/cm^2/hr), or apparent flux, as a function of time, was determined as the mean ± standard deviation of 3 to 9 individual measurements.

RESULTS

Figure 1 compares the anodal and cathodal iontophoresis of two bases (uracil and adenine) and two nucleosides (uridine and adenosine) with the corresponding passive transport. For these predominantly unionized species, anodal iontophoresis was more effective than cathodal, as expected from the known permselectivity of the skin (6), and the imposed direction of electroosmotic flow, which accompanies iontophoresis (12). The relative anodal and cathodal fluxes reflect, presumably, differences in the degrees of ionization of the different compounds. The maximum anodal fluxes for these four compounds fell in the range 1–2 nmol/cm^2/hr. Following current termination, the transport rates attained new passive levels (which were independent of whether anodal or cathodal iontophoresis had been applied) that were significantly greater than the control fluxes. This iontophoretically-induced increase in passive skin permeability has been previously observed for a variety of permeants, under comparable experimental conditions, to those employed here [e.g., 8].

The apparent passive and cathodal iontophoretic delivery profiles (as measured by the penetrated quantity of radiolabel) of the four nucleotides, from donor solutions of 1 mM concentration, are shown in Figure 2. The passive fluxes were negligible. In contrast, electrotransport rates were, on average, on the order of 2.5–3 nmol/cm^2/hr. Termination of current flow resulted in a quite rapid decrease in transdermal permeation.

The apparent iontophoretic delivery of AMP and ATP as a function of the initially applied concentration was found to be very linear (Figure 3). However, the steepness of the concentration dependence was greater for ATP than for AMP. For example, while a driving concentration of 20 mM AMP was necessary to achieve a maximal flux of ~40 nmol/cm^2/hr, the same apparent rate for ATP was reached with only 10 mM nucleotide initially present in the cathodal chamber.

Using the luciferase-luciferin assay, the degradation of ATP and GTP both in the donor electrode chamber, and during passage through the skin, was determined. The upper panels of Figure 4 show that essentially none of the permeated radioactivity (with or without iontophoresis) represented intact nucleotide. It was also found that 12 hours of iontophoresis, followed by 12 hours of passive diffusion, resulted in about 30% and 45% degradation of ATP and GTP, respectively, in the driving cathodal chamber. Similar experiments using ATP and fresh human skin were also performed (Figure 4, lower panels). While the flux of radioactivity across the human tissue was essentially identical to that through hairless mouse skin, the luciferase-luciferin assay again showed that none of the permeated radiolabel represented intact ATP, and that, again, significant degradation of the nucleotide occurred in the electrode chamber during the course of the experiment. Increasing the concentration of

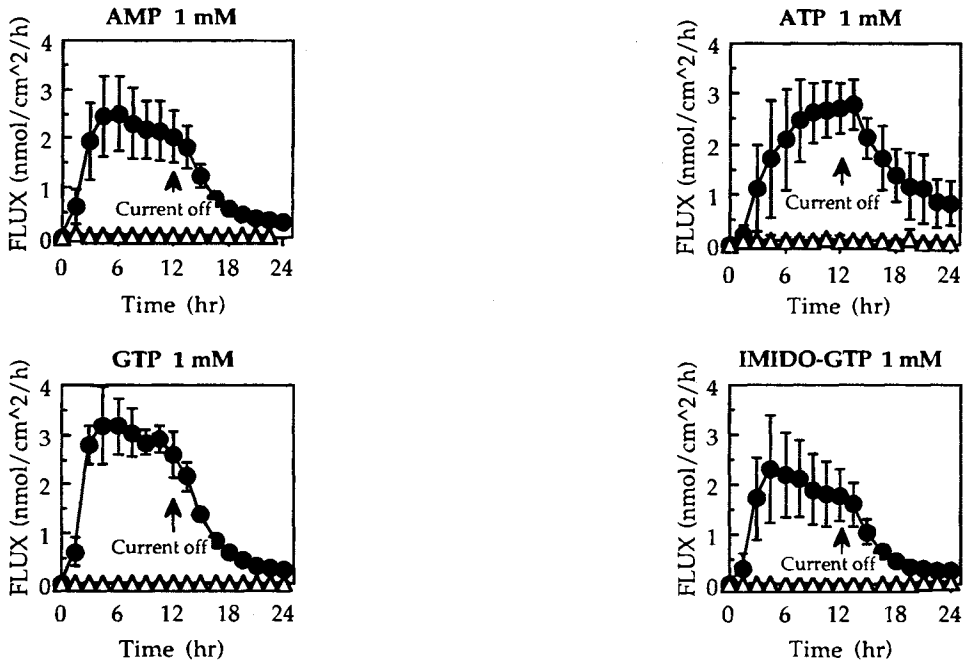

Fig. 2. Apparent fluxes of AMP, ATP, GTP, and imido-GTP across hairless mouse skin *in vitro,* as a function of time, following either iontophoretic (12 hours of constant current [0.55 mA/cm^2] from the cathode, closed circles) or passive (open triangles) delivery. Each data point represents the mean ± SD of 3–9 measurements.

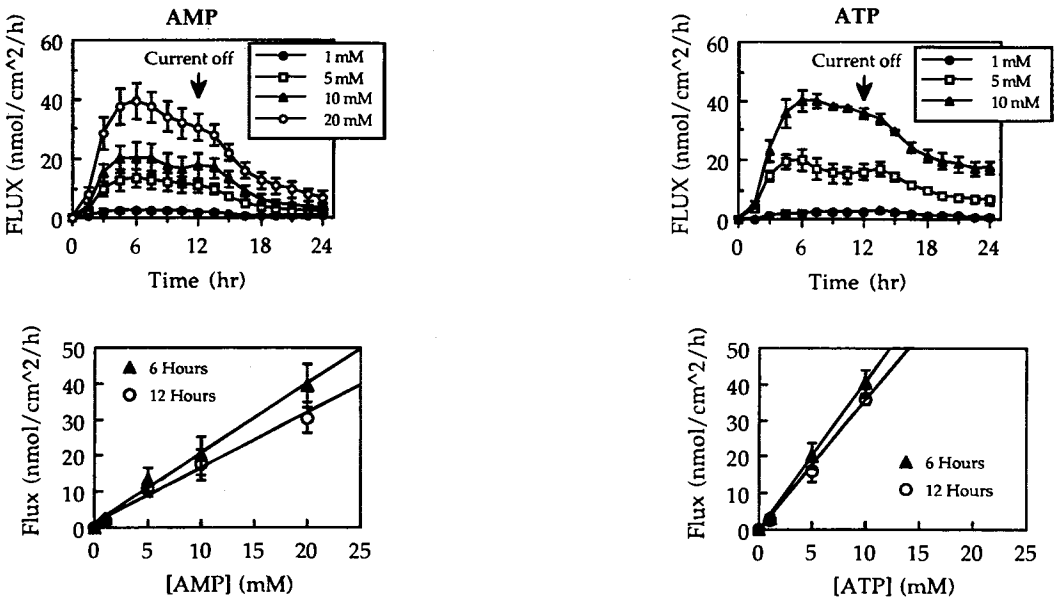

Fig. 3. Apparent fluxes of AMP and ATP across hairless mouse skin *in vitro,* when applied at different initial concentrations. The upper panels show the flux profiles as a function of time following iontophoretic (12 hours of constant current [0.55 mA/cm^2] from the cathode) delivery. The lower panels plot the measured iontophoretic fluxes after either 6 or 12 hours of current passage, as a function of the initial concentration applied. Each data point represents the mean ± SD of 3–9 measurements.

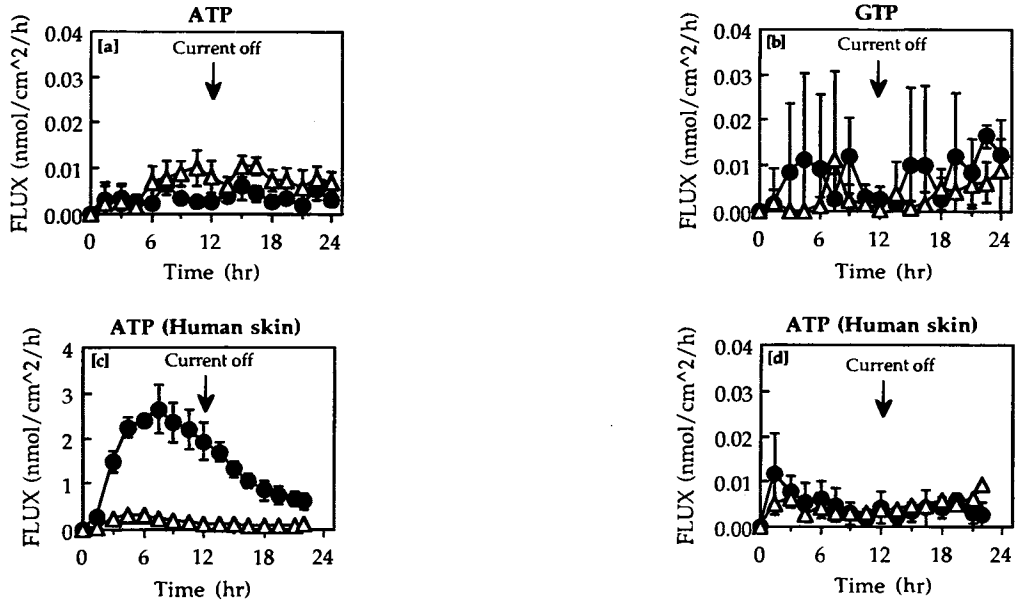

Fig. 4. (a) Actual flux of ATP across hairless mouse skin *in vitro,* as a function of time, following either iontophoretic (closed circles) or passive (open triangles) delivery. A specific assay for the intact triphosphate was used. (b) Actual flux of GTP across hairless mouse skin *in vitro,* as a function of time, following either iontophoretic (closed circles) or passive (open triangles) delivery. A specific assay for the intact triphosphate was used. (c) Apparent flux of ATP (as determined from the permeating quantity of radiolabel) across *human* skin *in vitro,* as a function of time, following either iontophoretic (closed circles) or passive (open triangles) delivery. (d) Actual flux of ATP across *human* skin *in vitro,* as a function of time, following either iontophoretic (closed circles) or passive (open triangles) delivery. A specific assay for the intact triphosphate was used. In all situations, the triphosphates were iontophoresed from the cathode for 12 hours at 0.55 mA/cm^2; each data point represents the mean ± SD of 3–9 measurements.

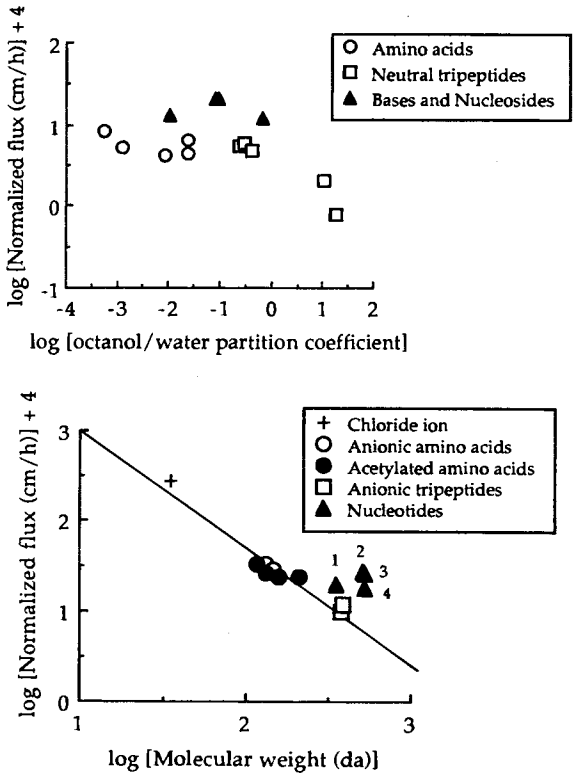

Fig. 5. Upper panel: 'Normalized flux' (defined as the *anodal* iontophoretic flux (in nmol/cm^2/hr) at 12 hours divided by the initially applied concentration (in nmol/cm^3)) for several uncharged compounds plotted as a function of lipophilicity (as measured by the corresponding octanol-water partition coefficient, P). Results from this study are compared with those previously reported by Green *et al.* (8, 9). Lower panel: 'Normalized iontophoretic flux' of several anionic compounds plotted as a function of molecular weight. Results from this study are compared with those previously reported by Green *et al.* (8, 9). Key: 1 = AMP, 2 = ATP, 3 = GTP, 4 = Imido-GTP.

ATP to 20 mM in the donor solution did not alter the quantity of intact compound permeating through to the receptor phase.

The selection of imido-GTP as one of the nucleotides for study was based upon its greater stability to hydrolytic degradation. The transport profiles for imido-GTP and GTP (Figure 2) were very similar, and the cumulative amounts permeating at 12 hours were not significantly different ($p > 0.05$). To determine whether the imido-GTP was stable during iontophoresis, the donor and receptor solutions were concentrated by lyophilization, reconstituted and subjected to thin layer chromatography. Although no degradation products were observed, the high salt concentration in the freeze-dried material precluded completely unequivocal chromatography. Therefore, the samples were subjected to the luciferase-luciferin assay using the approach that had been followed for GTP. In no case was it possible to transfer a phosphate to the added ADP, supporting the stability of imido-GTP under the conditions of the iontophoresis experiment.

DISCUSSION

The results in Figure 1 for the iontophoresis of the essentially uncharged bases and nucleosides are completely consistent with the earlier findings of Green *et al.* (7, 8), who studied the electroosmotic transport of several zwitterionic amino acids, and a number of neutral tripeptides, under essentially identical conditions. This parallelism is nicely illustrated by the upper panel of Figure 5, in which the previously published data are compared with those from this study. The Figure plots 'normalized flux' (defined as the *anodal* iontophoretic flux (in nmol/cm^2/hr) at 12 hours divided by the initially applied concentration (in nmol/cm^3) as a function of permeant lipophilicity (as measured by the compound's octanol-water partition coefficient, P). The results for the two bases and for the two nucleosides reported here are of the same order as those determined by Green *et al.* (7, 8) for the neutral amino acids and tripeptides. The striking lack of dependence upon log P confirms the earlier deduction that the transport environment in iontophoresis is *not* lipophilic.

Iontophoretic delivery of the negatively-charged nucleotides resulted in clearly different profiles of flux *versus* time (Figure 2). Although later investigation revealed that significant degradation of ATP and GTP was taking place during the course of the experiments, the patterns of transport nevertheless remained consistent with the movement of charged moieties (relatively rapid increase to maximal levels, which were higher than those observed for the essentially neutral bases and nucleosides). Furthermore, the fact that the results for AMP and ATP did not superimpose suggested that, in the ATP experiment, at least part (though probably not all) of the material crossing the rate-limiting barrier was the intact triphosphate. Once again, the general observations made with these negatively-charged permeants were very comparable to those reported for anionic amino acids (aspartic and glutamic acids) (7) and tripeptides (Ala-Asp-Ala and Ala-Glu-Ala) (8). The slight diminution in flux after the initial maximum is probably due to the progressively increasing amount of chloride available in the cathode chamber to compete with the negatively-charged permeant (8) or, perhaps, to a growing amount of phosphate originating from hydrolysis of the permeants. The lower panel of Figure 5 compares the normalized fluxes of the nucleotides, as an arbitrary function of molecular weight, with those of other anions which have been measured under closely similar conditions (8). Although all the nucleotides lie above the original linear regression of the data, there is certainly a consistency to the behavior observed. It should be emphasized that the initial dataset comprised exclusively singly-charged species, whereas the nucleotides are, of course, multiply-charged—this may explain, in part, why the results from this study lie systematically above the original regression.

The iontophoretic delivery of AMP was a linear function of the initial nucleotide concentration in the driving cathodal chamber (Figure 3). When ATP was used, an even greater apparent sensitivity to concentration was found, once again suggesting (a) that intact triphosphate was crossing the major resistance to iontophoretic delivery, and (b) that the level of charge on the permeant is an important parameter determining the efficiency of electrotransport. Figure 4, however, reveals that, if indeed ATP and GTP do cross into the viable skin tissue, they are then rapidly, and essentially completely, degraded. The totality of the biotransformation is independent of the source of skin employed. The more encouraging findings with imido-GTP suggest, however, that chemical modifications of nucleotide structures can be used to afford metabolic protection. That

being said, one is nevertheless cautioned not to extrapolate the present *in vitro* results to the *in vivo* situation. Qualitatively, similar rules may apply, but no guidelines exist for quantitative predictions of any kind.

In summary, then, this investigation has shown that the iontophoretic delivery of bases, nucleosides and nucleotides is possible. AMP is efficiently electro-transported across the skin from the cathode, and the flux can be linearly manipulated by the applied concentration. The triphosphates ATP and GTP also appear to be deliverable but significant and efficient degradation is apparent. On the other hand, a chemically-modified derivative, imido-GTP, appears to be more stable to electrochemical and/or metabolic breakdown. Thus, it remains to be seen whether iontophoresis offers a strategy for the delivery of oligonucleotides; certainly, the concept is beginning to attract attention, as exemplified by the recent paper of Oldenburg *et al.* (13), who examined the effects of salt concentration, pH, current density, oligonucleotide length, composition and concentration on the rate of electrotransport. While some of the results from this extensive study were consistent with expectations (e.g., flux increased with applied concentration and current density, but decreased with oligonucleotide length), others were less intuitively obvious, such as the dependence of iontophoretic delivery on base composition and oligonucleotide sequence (and/or conformation). Other investigators, recognizing the challenges associated with oligonucleotide transport, even with direct current, have considered electroporation (14, 15), and have reported (in the sense of enhanced delivery) positive results. Nevertheless, despite the recent burst of activity, it is clear that a systematic examination of the factors which determine the rate and extent of electrically-assisted oligonucleotide transport must be undertaken to fully delineate the opportunities and limitations of this novel approach to drug delivery.

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