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Action of Steroidal Diamines on Active Transport and Permeability Properties of *Escherichia coli*

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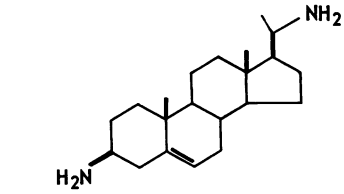
The steroidal diamine irehdiamine A (IDA) is a potent inhibitor of bacteriophage growth and macromolecular synthesis in *Escherichia coli*. By using radioactive ^{42}K and ^{14}C-thiomethylgalactoside (TMG), rapid effects of IDA and related steroids, both on the influx of potassium and TMG via their respective transport systems and on the efflux (leakage) of radioactivity from the treated cells, have been measured. IDA affects both the influx and efflux of ^{42}K at concentrations of steroid as low as 2×10^{-5} M. Because of the increased leakage, it is not possible to tell whether there is a direct effect reducing the rate of active transport of potassium. The primary diamine, IDA, and its bis-secondary, bis-tertiary, and bis-quaternary diamine analogues are decreasingly effective in altering cell permeability properties in the order $1° > 2° > 3° > 4°$. The effects of IDA on potassium transport are mirrored by similar effects on the transport of TMG. Therefore, the action of IDA is on the cell membrane and not directly on one or another transport system. The effects of IDA on cell permeability can reasonably explain the inhibitory actions of the drugs on bacteriophage growth and cellular metabolism.

Mahler and Baylor (15) recently described the inhibitory actions of the steroidal diamines irehdiamine A (IDA) and malouetine (MAL) on the growth of bacteriophage T2 in infected cells of *Escherichia coli*. Bacteriophage-directed deoxyribonucleic acid (DNA) synthesis is more sensitive to steroidal diamine inhibition than ribonucleic acid (RNA) or protein synthesis, and Mahler and Baylor (15) proposed that DNA is the primary target of steroidal diamine action. Because of the well-known interactions of polyamines and steroids with biological membranes (1, 18, 20, 26, 28) and because of our own interest in virus and chemically induced changes in the bacterial cell membrane (23-25), we have looked at the effects of steroidal diamines on cell permeability and active transport. To distinguish between highly specific effects on particular active transport systems and more general effects on the cell membrane, we have used two very dissimilar materials, potassium and thiomethylgalactoside (TMG), which share the common property that they are transported into bacterial cells against concentration gradients by means of active transport systems or "permeases" (9). The two active transport systems apparently are not closely related (5, 9, 12, 21). These experiments have led us to the conclusion that the primary attack of steroidal diamines is not on DNA but on the cell membrane, resulting in a generalized increase in cell permeability, so that small materials leak out of the cells, and in an inactivation of cellular permeases.

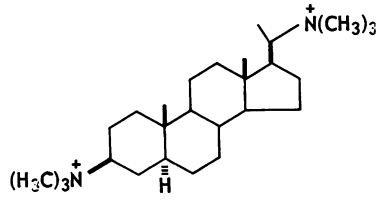
MATERIALS AND METHODS

E. coli strain BB was grown and studied in a dilute broth containing (per liter): 2 g of Tryptone and 1.25 g of NaCl. Dilute media are required for effective action of IDA on bacterial growth, as was reported by Mahler and Baylor (15) and confirmed in control experiments which showed little effect of IDA on potassium flux in full-strength broth (8 g of Tryptone per liter and 5 g of NaCl per liter). The physical basis for this dependency on dilute media is not fully understood, but the magnesium concentration plays a large role in determining the efficacy of IDA treatment (24*a*).

Steroidal diamines. IDA (pregn-5-ene-3β,20α-diamine), MAL [5α-pregnan-3β,20α-ylenebis(trimethylammoniumiodide)], and bis-monomethyl-, bis-dimethyl-, and bis-trimethyl-amino-substituted IDA were obtained from H. R. Mahler and M. B. Baylor. These plant alkaloids were originally obtained from the laboratories of Janot and Goutarel at the Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France, where they were purified and identified (7, 8, 10, 27). Both synthetic and natural IDA were used in these experiments. Although the synthetic material appears much less soluble than the natural product, the actions of the two products on potassium transport are indistinguishable. The molecular structures of IDA and MAL are shown in Fig. 1.



IREHDIAMINE A (IDA)



MALOUETINE

FIG. 1. *Molecular structures of irehdiamine A and malouetine.*

Radioisotopes. Spectrographic grade potassium chloride (^{42}K) was obtained from Iso/Serve, Inc., Cambridge, Mass. β-Methyl-^{14}C-thiogalactoside was obtained from the New England Nuclear Corp., Boston, Mass., and from Schwarz BioResearch Inc., Orangeburg, N.Y.

Radioisotope flux was measured as previously described (25). For the *efflux* experiments, *E. coli* BB was grown in dilute broth for several generations at 37 C with aeration by shaking, until the cells reached a density of about 5×10^8/ml. In the potassium experiments, ^{42}K was added during this growth period; the cells were collected by centrifugation at 20 C and were resuspended in fresh broth at 25 C. At this stage, essentially all of the radioactivity was in the cells and none was free in the medium. The cells were distributed in a series of small flasks; the flasks were placed in a shaking water bath at room temperature (23 to 29 C); and a 1-ml sample of each was filtered through Millipore HA filters (Millipore Filter Corp., Bedford, Mass.) and marked 0-min samples. Steroidal diamines were added and additional 1-ml samples were removed and filtered during the course of 1 hr at 23 to 29 C. The filters were not washed, because washing does not appreciably affect the quantitative results. The filtrates were collected in small "polyvials" (Olympic Plastics Co., Los Angeles, Calif.) and both the filters and samples of the filtrates were dried and counted in a Nuclear-Chicago low-background gas-flow counter. After corrections for the differences in geometry between filter and filtrate samples (measured directly in each experiment) and the decay of ^{42}K ($t/2$ = 12.4 hr), the data were prepared as percentages of the radioactivity remaining on the filters. The more accurate measurements of the remaining cellular radioactivity came from the filtrates and not from the filters, but the data used are the averages of the values from the filters and those from the filtrates.

The experiments with ^{14}C-TMG efflux were similar to those with ^{42}K, except that they were carried out in a cold room at 4 C instead of at ambient temperature. [This was necessitated by the relatively more rapid flux rates for ^{14}C-TMG than for ^{42}K (*see* 9, 12, 25) under otherwise comparable conditions of temperature, energy supply, and concentration. When ^{14}C-TMG flux experiments were attempted at 25 C, equilibrium was reached between intracellular and extracellular TMG before the first few samples could be taken.] *E. coli* BB was induced for the β-galactoside permease (9) during growth at 37 C by the addition of 10^{-4} M nonradioactive TMG, a gratuitous inducer for the β-galactoside system (from Sigma Chemical Co., St. Louis, Mo.), at least 1 hr before the cessation of growth. The cells were collected by centrifugation at 4 C and were resuspended in fresh dilute broth at 4 C. ^{14}C-TMG was added at 10^{-5} M (0.025 to 0.050 μc/ml) and the cells were allowed to accumulate radioactivity at 4 C for 1 hr. The cells were collected by centrifugation at 4 C and resuspended in fresh dilute broth at 4 C. After distributing the cells in a series of small flasks, samples were filtered and steroidal diamines were added as in the ^{42}K experiments, except that all incubation and filtering was at 4 C.

For measurements of rates of uptake of radioactivity, cells were grown at 37 C, as above, except that no radioactivity was added. For TMG influx experiments, the cells were induced by the addition of ^{12}C-TMG as in the efflux experiments. The cultures were cooled to 23 to 29 C (for ^{42}K) or 4 C (for ^{14}C-TMG). The β-galactoside permease-induced cells were centrifuged to remove the nonradioactive TMG and resuspended in fresh dilute broth at 4 C. Steroidal diamines were added either 5 min before or 1 min after the addition of ^{42}K or ^{14}C-TMG (time zero). Samples (1 ml) were removed, filtered, and washed two times with 5 ml of dilute broth at 25 or 4 C. In the influx experiments, only the filters were counted in the gas-flow counter.

RESULTS

IDA caused a rapid loss of cellular potassium. When IDA was added to cells prelabeled with radioactive ^{42}K, a rapid loss of radioactivity from the cells was seen (Fig. 2). Over the same range of concentrations where growth-inhibition is first observed, both the initial rate of loss and the ultimate "plateau" level depend on the concentration of IDA added. Although IDA caused this rapid loss of ^{42}K, its secondary diamine analogue, bis-monomethyl IDA, did not cause accelerated leakage at 5×10^{-5} M (Fig. 2). The secondary diamine did cause accelerated leakage, however, at higher concentrations (Fig. 4).

IDA caused a rapid loss of cellular TMG. Another experiment was similar to that in Fig. 2, except that the cells were preloaded with ^{14}C-TMG instead of ^{42}K, and the entire experiment was carried out at 4 C (Fig. 3). The rate of loss of ^{14}C-TMG at 4 C was similar to the rate of loss

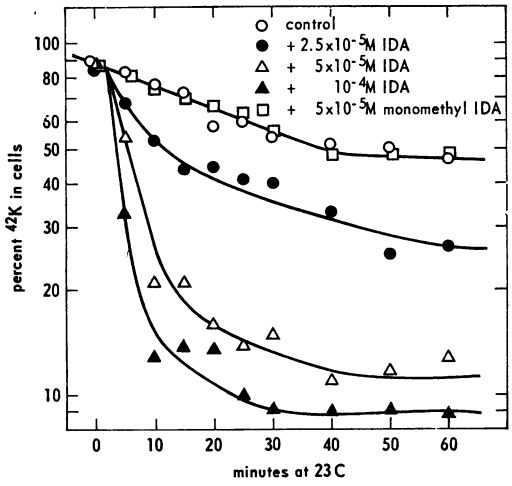


FIG. 2. *Steroid-induced leakage of radioactive potassium. Escherichia coli BB was grown in dilute broth containing ⁴²K, centrifuged, and resuspended in fresh dilute broth. Samples were removed and filtered as indicated. Steroidal diamines were added at 3 min.*

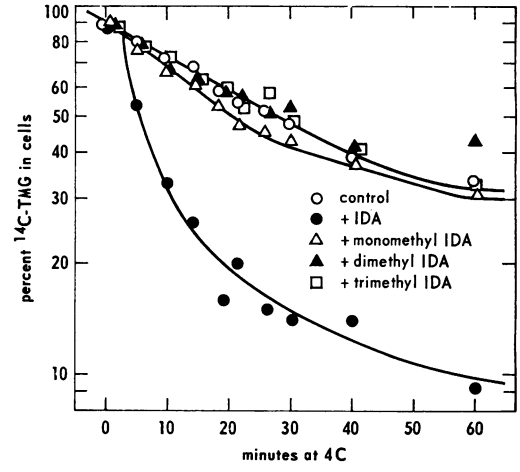


FIG. 3. *Steroid-induced leakage of ¹⁴C-thiomethyl-galactoside. Steroids were added at 10^{-4} M at 3 min.*

of ⁴²K at 23 C. This result was somewhat misleading, since the spontaneous rate of loss of radioactivity in both cases depended upon the external concentration of potassium or TMG (4, 5, 9, 12; Silver, *in preparation*) and, in the TMG experiment, there was no added nonradioactive TMG, whereas, in the potassium experiments, the broth contained about 1.5×10^{-4} M potassium. Nevertheless, comparisons of Fig. 2, 3, and 4 demonstrate why it was necessary to perform the TMG flux experiments in the cold. As in the case of potassium leakage, IDA caused a rapid loss of cellular TMG. The amino-substituted diamines had little or no effect on TMG efflux (*see below*).

Specificity with regard to steroidal diamine structure. Mahler and Baylor (15) observed that IDA is a more potent inhibitor of bacteriophage growth than is MAL. Mahler et al. (17) found differences in the effects of IDA and MAL on the secondary structure of DNA. In these physical-chemical studies, it could be shown that the significant difference between IDA and MAL was not the presence or absence of the double bond in the B-ring (Fig. 1), but the distinction between a primary diamine (IDA) and a quaternary diamine (MAL). This was demonstrated by using analogues of IDA with the same ring structure but substituted amino groups. In analogous experiments measuring potassium efflux, bis-trimethyl IDA, which differs from MAL only with regard to the 5,6 double bond, was no more effective than MAL in increasing potassium leakage (Fig. 4). The bis-dimethyl IDA was effective in inducing a higher rate of loss of radioactive potassium, and the bis-monomethyl IDA was more effective than the bis-dimethyl IDA but much less effective at the same concentration than was the unsubstituted primary diamine IDA (Fig. 4). With the bis-monomethyl IDA, an effect on potassium efflux was seen when the concentration was raised from 5×10^{-5} M (Fig. 2) to 10^{-4} M (Fig. 4). MAL, the quaternary diamine, had no effect on potassium efflux at concentrations as high as 3×10^{-4} M (*unpublished data*). The effects of the series of substituted diamines on ¹⁴C-TMG efflux were measured in the experiment shown in Fig. 3. IDA caused accelerated loss of ¹⁴C-TMG, bis-monomethyl IDA had a barely detectable effect on ¹⁴C-TMG efflux, and bis-dimethyl and bis-trimethyl IDA were without effect when the steroidal diamines were added at 10^{-4} M (Fig. 3).

Both IDA and MAL inhibited the influx of ⁴²K into bacterial cells. In this experiment (Fig. 5), the steroidal diamines were added 5 min prior to the addition of ⁴²K. At 10^{-4} M, IDA inhibited the accumulation of ⁴²K by the cells more than 98%, but, because of the leakage induced by IDA, we were not sure whether this was a direct effect on the potassium influx or whether the influx continued unabated but the cells lost potassium as rapidly as they could accumulate it (*see also* Fig. 7). The results in Fig. 5, together with those in Fig. 2 and 4 showing accelerated efflux of potassium in the presence of IDA, establish that the cells suffered a rapid net loss of potassium. The MAL data (Fig. 5) show a direct effect on potassium influx because we have already seen (Fig. 4) that MAL does not cause accelerated leakage of potassium. In the

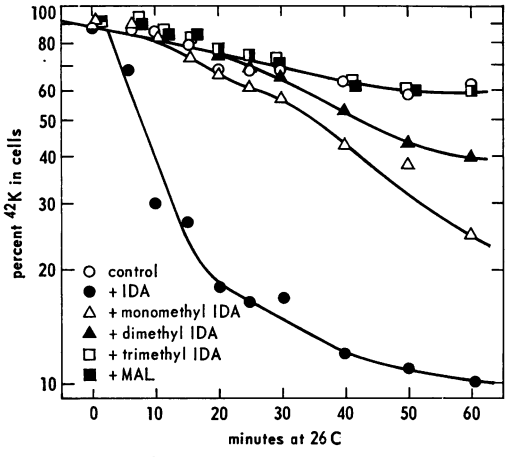


FIG. 4. *Specificity of steroid-induced potassium leakage. The experiment was similar to that in Fig. 2 except that the water bath was held at 26 C. Steroidal diamines were added at 10^{-4} M at 3 min.*

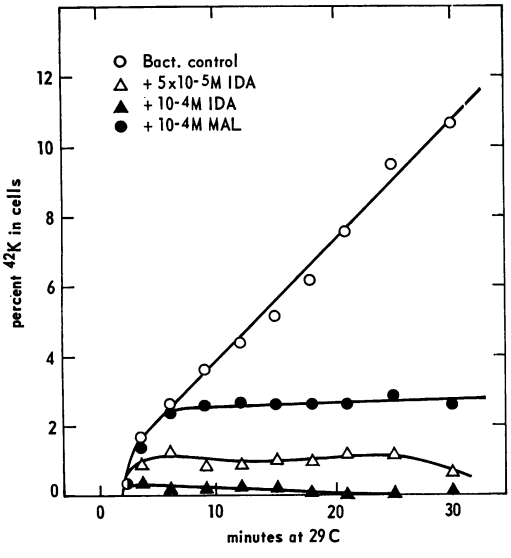


FIG. 5. *Steroid inhibition of potassium influx. Steroids were added to nonradioactive E. coli at 0 min and ^{42}K (0.05 μc/ml; 1.3 × 10^{-5} M potassium) was added at 2 min. The ordinate refers to the percentage of total radioactivity retained on the filter.*

presence of 10^{-4} M MAL, with a normal rate of efflux and a reduced rate of influx, there is also a net loss of cellular potassium.

The experiments designed to measure the effects of the substituted diamines on potassium influx gave results complementary to those from the potassium efflux experiments. At low concentrations of steroidal diamines (5 × 10^{-5} M), IDA effectively inhibited the influx of potassium,

TABLE 1. *Specificity of steroid inhibition of potassium influx*[a]

Steroidal diamine	Initial rate	^{42}K in cells at 30 min
	%/min	%
Control	0.53 (100)	7.0 (100)
+ IDA	0.08 (15)	0.07 (1.0)
+ Monomethyl IDA	0.26 (49)	0.26 (3.7)
+ Dimethyl IDA	0.47 (88)	2.6 (37)
+ Trimethyl IDA	0.50 (94)	3.1 (44)

[a] Steroidal diamines at 1.5 × 10^{-4} M were added to cells of *E. coli* 3 min before the addition of ^{42}K (0.03 μc/ml; 1.7 × 10^{-6} M) in dilute broth at 25 C. Samples were removed, filtered, and washed two times with 5 ml of dilute broth, and the filters were counted. The initial rate of influx was determined from samples filtered after 3 min. Numbers in parentheses are percentages of control values.

but the monomethyl, dimethyl, and trimethyl-substituted IDA compounds had little or no effect (*unpublished data*). At higher concentrations (10^{-4} M or more), the substituted diamines began to show inhibitory effects on potassium influx (Fig. 5; Table 1). Again, there is a gradient of efficacy of inhibition by the diamines: 1° > 2° > 3° > 4°.

Both IDA and MAL inhibit the influx of ^{14}C-TMG into bacterial cells. Greater concentrations of steroids were needed to accomplish inhibition similar to that seen with ^{42}K (Fig. 6). However, higher concentrations of dinitrophenol and cyanide are also required to inhibit TMG influx than to inhibit potassium influx (12; *unpublished data*). IDA accomplished a similar fractional reduction in the rate of influx of TMG (Fig. 6) at less than one-tenth the concentration of cyanide (compare the data for 10^{-4} M IDA with that for 10^{-3} M NaCN). MAL appeared to be about as effective as cyanide on a mole-for-mole basis in inhibiting TMG influx (Fig. 6). These conclusions have been confirmed in additional ^{14}C-TMG experiments, both at 4 C and at 26 C, and appear to be approximately true also for the relative effects of IDA, MAL, and cyanide on potassium influx (*unpublished data*).

Effect of IDA on potassium transport is reversible. An experiment (Fig. 7) was designed to determine whether the effect of IDA on potassium influx (as in Fig. 5) is reversible. Control data (Fig. 7a) show normal influx of ^{42}K and the inhibition of influx caused by 2 × 10^{-5}, 4 × 10^{-5}, and 10^{-4} M IDA. There was measurable inhibition of ^{42}K influx at concentrations as low as 2 × 10^{-5} M IDA. In this experiment, the IDA was added after and not before the addition of ^{42}K, so

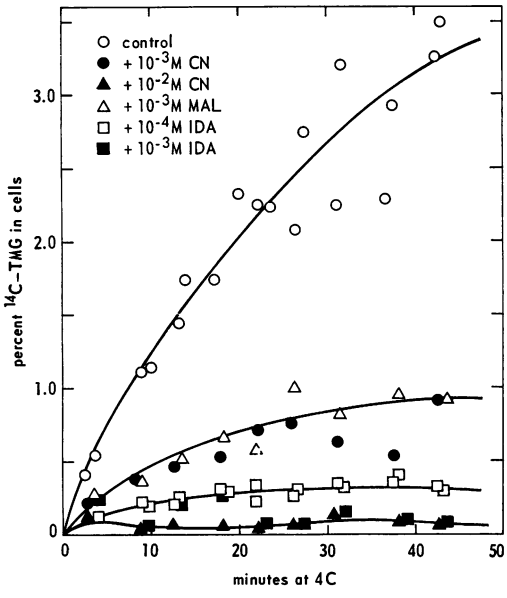


FIG. 6. *Steroid inhibition of ^{14}C-thiomethylgalactoside (TMG) influx. Steroids or NaCN were added at −5 min at 4 C. ^{14}C-TMG (0.05 μc/ml; 10^{-5} M) was added at 0 min. The ordinate refers to the percentage of total radioactivity retained on the filter.*

that the effect of the addition of IDA was discernible in less than 2 min at 29 C and the ^{42}K (which entered the cells prior to the addition of IDA) leaked out after the addition of IDA (Fig. 7a). For this reason, we are not sure whether there is a direct effect of IDA on the potassium influx. Comparable data were obtained on potassium influx. from cells which were pretreated with 10^{-4} M IDA for a 15-min period, centrifuged, and resuspended in fresh medium for a 20-min "recovery" period prior to the addition of ^{42}K (Fig. 7b). By comparing the control curves in Fig. 7a and 7b, one can see a 70% recovery for both the initial rate of ^{42}K uptake and the amount of ^{42}K in the cells after 30 min. The cells which have been pretreated with IDA and "recovered" are still sensitive to an additional treatment with IDA (Fig. 7b).

When the time of pretreatment with 10^{-4} M IDA was varied from 0 to 60 min and the recovery period was held constant at 25 min at 25 C, the irreversible inactivation of the ability to concentrate ^{42}K occurred with a half-life of about 30 min at 25 C (Table 2). The results were approximately the same whether we used the initial rate of uptake or the 30-min data (approximately steady-state equilibrium between intra- and extracellular ^{42}K). Therefore, the 15- to 25-min recovery period allowed maximal recovery of the ^{42}K active transport system in those cells which were capable of such recovery. Experiments are in progress to measure the kinetics of this recovery process and to determine whether energy utilization or protein synthesis is required for recovery (24*a*).

DISCUSSION

Mode of action of IDA and MAL. We have shown a rapid effect of IDA on the efflux of ^{42}K and ^{14}C-TMG from cells treated with concentrations as low as or lower than those required to inhibit bacterial and bacteriophage growth. Low concentrations of IDA also prevent the cellular accumulation of potassium and galactosides. However, because of the rapid rate of leakage in the presence of IDA, we cannot be sure whether IDA is acting in two separate ways, one causing the leakage and another acting directly on the active transport mechanisms, or whether the excessive leakage prevents the cell from accumulating materials even though the active transport systems continue functioning. From the experiments showing inhibition of potassium and TMG influx after MAL treatment, we concluded that there is a direct effect on the active transport systems because there is no indication of increased leakage or turnover after exposure to MAL. Therefore, either MAL and IDA have distinct modes of action in inhibiting bacterial active transport, or both IDA and MAL must have direct effects on the permease systems. This remains an unresolved question.

Mahler and Baylor (15) postulated a direct effect of IDA on the DNA within the phage-infected cells. We have little evidence for or against this hypothesis. However, it is unnecessary to invoke a direct effect of IDA binding to DNA to satisfactorily explain Mahler and Baylor's experimental results, and the low ionic-strength conditions required for IDA binding to polynucleotides in vitro (14, 17) would be found in the cells only after the loss of potassium and magnesium by excessive leakage (4, 22; Silver, *in preparation*).

In addition to the evidence for IDA binding to polynucleotides in vitro, the principle basis for Mahler and Baylor's (15) conclusion that there is a direct effect of IDA on DNA is the observation that, in phage-infected cells, phage-directed DNA synthesis is more sensitive to inhibition by IDA than is phage-directed RNA or protein synthesis. However, in uninfected cells, the syntheses of DNA, RNA, and proteins are about equally sensitive to IDA (15), and the growth of the RNA-containing virus MS2 is only slightly less sensitive to IDA inhibition

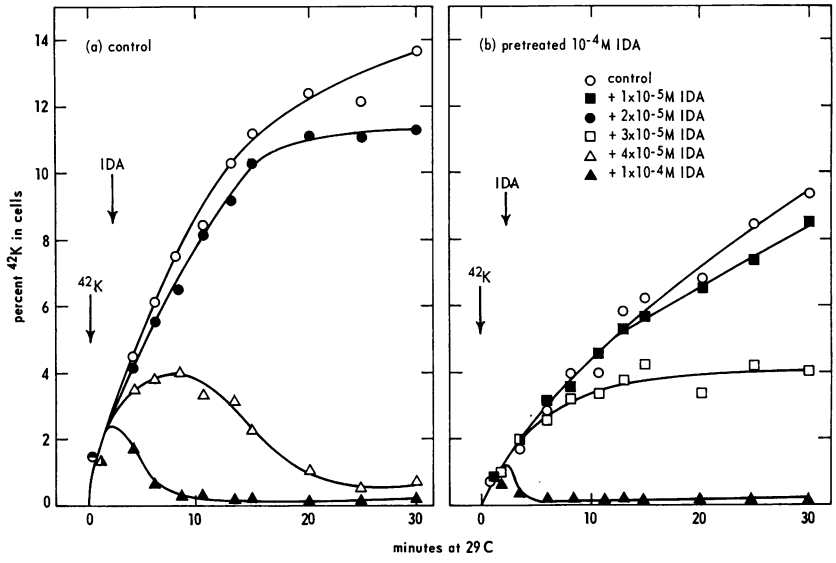


FIG. 7. *Reversibility of the action of IDA on potassium influx. (a) Control experiment was similar to that in Fig. 5. ^{42}K (0.01 μc/ml; 1.3 × 10^{-5} M potassium) was added at 0 min. IDA was added 2 min later. (b) Recovery experiment was similar to that in Fig. 7a, except the cells had been pretreated with 10^{-4} M IDA for 15 min at 25 C, centrifuged, resuspended, and allowed 20 min at 25 C for recovery prior to the addition of ^{42}K. The ordinate refers to the percentage of total radioactivity retained on the filter.*

TABLE 2. *Recovery from IDA treatment*[a]

Treatment	Initial rate		^{42}K in cells at 30 min	
	%/min		%	
Control	1.0	(100)	22	(100)
Pretreated with IDA				
15 min	0.7	(70)	17	(77)
30 min	0.5	(50)	13	(59)
60 min	0.2	(20)	5.4	(25)

[a] Cells of *E. coli* BB were pretreated with 10^{-4} M IDA for 0, 15, 30, or 60 min at 25 C, centrifuged, and resuspended in fresh dilute broth. After an additional 25-min recovery period at 25 C, ^{42}K was added and samples were removed, filtered, and washed twice with 5 ml of dilute broth. Both the initial rate of influx (which was constant for at least 10 min) and the relative amount of radioactivity in the cells after 30 min are shown. Numbers in parentheses are percentages of recovery relative to control values.

than is T2 growth. We would favor the following hypothesis: that the inhibition of DNA, RNA, and protein synthesis by IDA is either a secondary consequence of the loss of small pools of required ions and substrates by leakage or, more likely, that these inhibitory effects reflect the physical or chemical coupling of structure and synthesis of macromolecules to the cell membrane. The difference in sensitivity to IDA between uninfected and phage-infected cells would then be due to a difference in the membranous structure which is the site of DNA synthesis (6, 13, 19), rather than to a direct effect on DNA synthesis in phage-infected cells, as proposed by Mahler (*personal communication*). The inhibition of DNA synthesis by membrane alterations was discussed at some length with regard to the proposed mechanism of action of phenethyl alcohol (25). Phenethyl alcohol was initially thought to have a direct effect on DNA synthesis, but, after some experimental inconsistencies, such as inhibitory effects on the growth of RNA-containing phage, phenethyl alcohol was shown to cause alterations in cell membrane properties (25).

"Pharmacological" action of IDA. Both IDA and MAL are natural alkaloids which occur in a family of plants well known for neurologically active alkaloids (7, 10, 11). MAL, in fact, has been found to have a curare-like effect on animals (11). In contemplating that the effects of the steroidal diamines on cation transport might serve as an introduction to a "bacterial neurophysiology," we were tempted to measure effects of other curare-like drugs on bacterial potassium transport. Tubocurare, however, was without effect on potassium flux at concentrations as high as 3 × 10^{-4} M (*unpublished data*). Clayton (2) reported a small effect of curare at 10^{-2} M on phototaxis in *Rhodospirillum rubrum* and, at

5×10^{-3} M or higher concentrations, tubocurare inhibits potassium influx in *E. coli*. We do not believe this inhibition has the same degree of specificity as the IDA inhibition, because (i) it occurs only at concentrations of tubocurare high enough to cause massive clumping of cells and (ii) similar clumping can be obtained at these high concentrations by using other polyamines such as streptomycin, acriflavine, spermine, and spermidine, but not with putrescine or gallamine triethiodide (*in prepration*).

Reagents which interrupt bacterial growth and alter active transport or cell permeability, or both, can be grouped into three classes on the basis of effects on potassium transport. (i) Cyanide, which presumably acts by limiting the cell's energy supply (9, 16, 29), slows or eliminates the influx of potassium and very much slows the efflux of potassium. Therefore, over a short period of time (during which cellular growth and macromolecular synthesis are shut down), the bacterial cells retain a relatively constant internal concentration of potassium. MAL, with regard to potassium economy, has an effect similar to cyanide. (ii) IDA has a very different effect on active transport and permeability than does cyanide. IDA causes gross leakage of small molecules from the cells and may or may not directly affect the active transport system. (iii) Previous studies with phenethyl alcohol (25) have shown that accelerated leakage of potassium does not invariably lead to inhibition of the active transport systems. When phenethyl alcohol causes increased leakage of potassium from bacterial cells, the potassium influx system compensates, so as to retain a reasonably constant internal potassium concentration even in the face of the increased rate of turnover.

Reversibility of IDA-induced changes in membrane properties. We were surprised to see how readily reversible were the effects of IDA on potassium transport. Mahler and Baylor (15) reported cell killing with 10^{-4} M IDA. Under the conditions in Table 2, where the potassium transport system is inactivated with a half-life of about 30 min, the bacteria lose the ability to form colonies with a half-life of about 8 min (L. Hafken, *unpublished data*). We do not know the nature of the lethal event, but apparently it is not the irreversible inactivation of the potassium transport system. One way to distinguish between killing at the DNA level and killing at the level of the cell membrane would be to measure the detailed kinetics of killing. Killing at the level of the cell membrane might be expected to follow a simple exponential function, whereas killing at the DNA level would be expected to show a "shoulder," because of the multinucleate nature of the bacterial cells (e.g., 3). The kinetic data of Mahler and Baylor (15) do not allow a distinction between these alternatives for phage-infected cells; similar experiments with uninfected cells are currently in progress in this laboratory.

ACKNOWLEDGMENTS

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