# The Mode of Action of Levallorphan as an Inhibitor of Cell Growth

RICHARD GREENE[1] AND BORIS MAGASANIK

*Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139*

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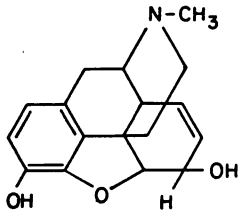
SUMMARY

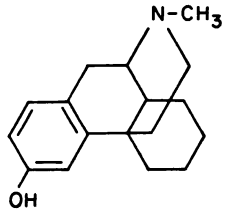
Levallorphan inhibits the synthesis of RNA, DNA, and protein by *Escherichia coli*. The drug was found to cause an immediate, dramatic decrease in the amount of intracellular ATP. Evidence is presented which suggests that the decrease in ATP concentration is a consequence of the stimulation of an ATPase. Similar results were obtained in HeLa cells treated with levallorphan.

## INTRODUCTION

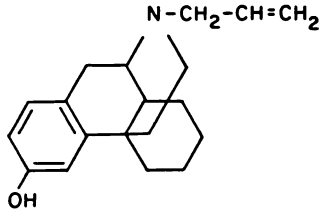
It has been reported by Simon (1) that several synthetic analogs of the morphine series are strong inhibitors of bacterial growth. Simon attributes this effect to selective inhibition of ribosomal RNA synthesis (2, 3). We have studied the effects of the most potent of these drugs, levallorphan tartrate, on macromolecular synthesis in *Escherichia coli*. This drug was selected for study because it is not classified as a narcotic and because it is more soluble than its narcotic analog, levorphanol.




Morphine




Levorphanol




Levallorphan

At a concentration of 5 mM, levallorphan rapidly stops the synthesis of RNA, protein, and DNA. Because we found that the synthesis of all macromolecules is stopped by levallorphan, we investigated the effect of this drug on energy metabolism. We find that the drug brings about the disappearance of intracellular nucleotide triphosphates.

A brief report of these results has been published (4).

## MATERIALS AND METHODS

*Chemicals.* Levallorphan tartrate and levorphanol tartrate were gifts of Hoffmann La Roche, Inc. L-Leucine-1-$^{14}$C, uracil-2-$^{14}$C and thymidine-2-$^{14}$C were obtained from New England Nuclear Corporation at activities of 27.8, 25.4, and 26.4 mC/mM, respectively. Guanine-8-$^{14}$C (32 mC/mM) was obtained from Schwarz BioResearch, Inc., and adenine-8-$^{14}$C (31.3 mC/mM) was purchased from Nuclear Chicago Corporation. Methyl-$^{14}$C-thiogalactoside (4 mC/mM) was obtained from New England Nuclear Corporation. Purine

[1] Present address, National Institutes of Health, Bethesda, Maryland 20014.

and pyrimidine nucleotides were obtained from Sigma Chemical Company. ONPG[2] and IPTG were purchased from Mann Research Laboratories, Inc. Polyethyleneimine was obtained from Chemstrand Corporation.

All other chemicals were of reagent grade and readily available from commercial suppliers.

*Bacteria.* A methionine-requiring mutant of *E. coli* K12, Hfr Cavalli, was obtained from Dr. S. E. Luria. This strain is a derivative of K12 58-161 (W-6) and has the $RC^{rel}$ character (5).

*Escherichia coli* AB 1105 was a gift of Dr. Loretta Leive. This strain is an auxotroph requiring proline, histidine, and thiamine and does not contain the β-galactoside permease ($i^+z^+y^-$).

*Bacillus subtilis* W-23 (wild type) was obtained from Dr. C. Levinthal.

*Media. Escherichia coli* was grown at 37° on a synthetic medium, pH 7.0, containing per liter: 21.0 g of $K_2HPO_4$, 9.0 g of $KH_2PO_4$, 0.1 g of $MgSO_4$, 2 g of $(NH_4)_2SO_4$, and 0.94 g of sodium citrate. Glucose, at a final concentration of 0.1% or sodium succinate at a final concentration of 0.5% were used as carbon sources. Amino acid requirements were supplied at a concentration of 20 μg/ml.

*Bacillus subtilis* W-23 was grown at 37° in the minimal medium described above with 0.1% glucose as major carbon source. Tryptone was added at a concentration of 0.1 mg/ml.

Cells were grown in flasks in a New Brunswick shaker. Bacterial growth was followed in a Klett-Summerson photoelectric colorimeter with a 420 mμ filter. Cells were used routinely at a concentration of 2 to $3 \times 10^8$ per milliliter.

*Incorporation of uracil-$^{14}C$, leucine-$^{14}C$, and thymidine-$^{14}C$.* Samples of 1 ml were taken at timed intervals from cultures in the exponential phase of growth, added to 1 ml of cold 10% trichloroacetic acid and allowed to stand for 30 min in the cold. The samples from the uracil-$^{14}C$ and thymidine-$^{14}C$ labeled cultures were filtered through Millipore filters (0.8 μ pores, Millipore Corporation, Bedford, Massachusetts) and washed with 5 volumes of cold 5% trichloroacetic acid. The filters were glued to planchets and counted in a low background, Nuclear Chicago gas flow counter. The samples labeled with leucine-$^{14}C$ were placed for 30 min in a water bath at 100° before being filtered and washed with trichloroacetic acid.

*Sucrose gradients.* Crude extracts of cells were prepared by grinding cells in the cold with alumina according to the method of Nakada *et al.* (6). Linear sucrose gradients from 5 to 20% were prepared and the crude extracts were layered on the gradients and run in a Spinco Model L ultracentrifuge as described by Nakada *et al.* (6). After centrifugation the tubes were punctured and drops were collected for the measurement of optical density at 260 mμ and for analysis of radioactivity. Some samples were pumped through a Gilford recording spectrophotometer which gave a continuous optical density reading of the gradient. Drops for radioactivity determinations were collected from these gradients after they had passed through the continuous-flow cuvette.

*Induction of β-galactosidase.* β-Galactosidase was induced in *E. coli* by addition of IPTG to the culture to a final concentration of $5 \times 10^{-4}$ M.

*Assay of β-galactosidase.* At timed intervals 1-ml samples were removed from the culture and pipetted into prechilled tubes containing one drop of toluene. The tubes were shaken vigorously and kept chilled until all samples had been collected. The activity of β-galactosidase was determined by the rate of hydrolysis of ONPG according to the method of Pardee *et al.* (7).

For permeability studies the rate of hydrolysis of ONPG by intact cells was measured. A preinduced culture in the exponential phase of growth was incubated for 2 minutes with 100 μg/ml of chloramphenicol to stop further synthesis of β-galactosidase. From this culture 1 ml was taken and added to 0.3 ml of 0.013 *M*

[2] Abbreviations used: ONPG = *o*-nitrophenyl-β-galactoside; IPTG = isopropylthio-β-D-galactoside; TMG = methylthio-β-D-galactoside; mRNA = messenger RNA.

ONPG. The change in optical density at 420 mμ was followed for 20 min in a Zeiss spectrophotometer.

*Measurement of mRNA decay in B. subtilis* (8). A culture of *B. subtilis* W-23 was given 0.1 μC/0.36 μg/ml of uracil-$^{14}$C. After 30 seconds actinomycin was added to the culture to a final concentration of 10 μg/ml. Aliquots of 1 ml were removed from the culture and added to an equal volume of cold 10% trichloroacetic acid, filtered and washed as described above. The filters were glued to planchets and counted in a low background counter.

*Measurement of ATP by the firefly method.* Determination of ATP concentration was done according to the method of Holt (personal communication) using crude extracts of firefly lanterns available from Sigma Chemical Company. To extract the nucleotides 1 ml of culture or 1 ml of medium filtered free of bacteria was added to 5 ml of $H_2O$ and placed in a boiling water bath for 10 min. Aliquots of these extracts were used in the final assay.

A luciferin-luciferase preparation (Sigma FLE-50), 50 mg in 5 ml of cold $H_2O$, was vigorously shaken for 2 min. The resulting suspension was centrifuged at 11,000 rpm at 5° for 35 min. The clear yellow supernatant solution was decanted and kept at 0° for use in the ATP assay.

The amount of light emitted by the reaction of ATP with this firefly extract was measured in a Beckman model DU spectrophotometer, previously adjusted to allow no stray light into the cuvette chamber. To a 3-ml cuvette 0.4 ml of distilled water was added and mixed with 0.2 ml of the firefly extract on a Vortex Jr. Mixer at low speed to avoid foaming. Exactly 15 sec later 0.2 ml of the boiled bacterial extract or culture fluid was added and mixed on the shaker for 5 sec. The percent transmittance of light was measured exactly 15 sec after addition of the boiled bacterial extract or culture fluid. The values so obtained were corrected for the amounts of light emitted when distilled water was added to the firefly extract. This blank varied considerably from extract to extract. This method gives linear results in the range of $10^{-7}$ to $10^{-9}$ *M* ATP. Other triphosphates (UTP, CTP, and GTP) are each about one-third as active as ATP, and ADP is about one-tenth as active as ATP.

*Oxygen consumption.* The rate of $O_2$ consumption was measured in a Warburg apparatus according to the method of Umbreit (9).

*Glucose metabolism.* The disappearance of glucose from a bacterial culture was measured enzymically by means of the *glucostat* reagent (Worthington Biochemical Corporation). This reagent contains the enzymes glucose oxidase and peroxidase which couple the oxidation of glucose yielding $H_2O_2$ to the oxidation of a chromogen. The enzyme and chromogen are supplied separately as solids. They are each dissolved in water and mixed to give a final volume of 90 ml. Four milliliters of this reagent was added to 1 ml of culture and allowed to stand at room temperature for 10 min. The reaction was stopped by adding one drop of 4 N HCl. The color developed was read in a Zeiss spectrophotometer at 400 mμ. Under these conditions a linear standard curve is obtained with known concentrations of glucose up to 0.2 mg/ml.

*Isolation and thin-layer chromatography (10) of nucleotides.* The nucleotides of *E. coli* K12 were labeled with adenine-$^{14}$C or guanine-$^{14}$C at a specific activity of 31.3 mC/mmole for one generation. The cells were then filtered, resuspended in fresh medium free of radioactivity, and treated with the drug for 10 min. The cells and medium were separated by filtration through a Millipore filter (0.45 μ pore size). The cells were resuspended to their original volume with cold medium. Both cells and medium were treated with one-half volume of 7% cold perchloric acid and filtered free of material insoluble in perchloric acid. The nucleotides in the resulting filtrate were adsorbed to 20 mg of acid-washed charcoal (Norite). The charcoal suspensions were shaken for several seconds in the cold, filtered on a Millipore filter (0.45 μ pore size), and washed with 5 ml of 1 mM HCl; the nucleotides were eluted with 4 ml of a solution of ethanol, M $NH_4OH$, $H_2O$ (66, 10, 133 by volume)

the pH of which was 11.3. The eluate was then evaporated to dryness using a filtered air stream. The resulting powder was resuspended in 0.2 ml of distilled water. The nucleotides in this solution were separated by thin-layer chromatography (TLC) according to the method of Randerath (10).

The TLC plates were prepared using a mixture of the anionic exchange resin polyethyleneimine (Polymin P, Chemstrand Corporation) and cellulose (Macherey and Nagel Company, MN300). A solution of 100 g of polyethyleneimine in 250 ml of water was adjusted to pH 6 with concentrated HCl and diluted to 500 ml with water. Aliquots of 20 ml were dialyzed against 4 liters of water for 24 hr. The contents of each dialysis bag were diluted to 200 ml with water. From this solution 130 ml was mixed with 20 g of cellulose powder in a Waring blendor for 30 sec, placed in the cold for approximately 30 min, and spread on thin plastic sheets by a TLC spreader Model No. 611 (Desaga Company, Heidelberg, Germany). The plates were allowed to dry overnight, and were then washed once with water. A solution of 10% NaCl was then allowed to ascend to 5 cm, after which the plates were developed with distilled water. The plates were then dried and spotted with the nucleotide solutions. Free bases and nucleosides were washed to the top of the plate by allowing water to ascend the entire height of the plate. The nucleotides were then separated by ascending chromatography with 1.1 M LiCl as the eluting solution. The chromatograms were dried and counted in a Nuclear Chicago strip counter. This method cleanly separates ATP, ADP, and AMP, but adenine nucleotides cannot easily be separated from guanine nucleotides. However, it was shown, by hydrolyzing the extracted nucleotides with 1 N $H_2SO_4$ at 100° for 1 hr and separating the resulting bases and nucleosides by electrophoresis in 0.03 M citrate buffer pH 4.1, that under the conditions of labeling with adenine-$^{14}$C more than 85% of the label in the extract resided in adenine nucleotides. Thus essentially all the counts on the TLC strip were in adenine nucleotides.

*HeLa cell studies.* HeLa cells were kindly supplied by Dr. Sheldon Penman. The cells were grown in spinner cultures on Eagle's medium with 7% horse serum. Studies of the incorporation of labeled compounds into protein and RNA as well as studies of changes in the nucleotide composition of the HeLa cell utilized essentially the same techniques as those described above for bacterial studies.

Incorporation of leucine-$^{14}$C and uridine-$^{14}$C into protein and RNA was studied by exposing cells to the labeled compound, removing 1-ml aliquots of the culture at timed intervals, and pipetting them into 4 ml of ice cold Earle's buffer. The cells were then collected by centrifugation and resuspended in 2 drops of water. One milliliter of cold 10% trichloroacetic acid was added, and the acid-precipitable material was collected on a Millipore filter (0.8 $\mu$) and washed with 5 volumes of cold 5% trichloroacetic acid. The filters were dried, glued to planchets, and counted in a low background counter.

The nucleotides of HeLa cells were labeled for 15 minutes with adenosine-$^{14}$C (210 mC/mmole). The cells were centrifuged and resuspended in medium without labeled adenosine but containing 3 mM levallorphan. After 10 min of drug treatment the cells were centrifuged and resuspended in their original volume of Earle's buffer, and their nucleotides were extracted and separated as described above for bacteria.

## RESULTS

### *The Effect of Levallorphan on Macromolecular Synthesis*

Figure 1 shows the effect of various concentrations of levallorphan on the synthesis of protein and RNA, measured by the incorporation into acid-insoluble material of leucine-$^{14}$C and uracil-$^{14}$C, respectively, by *E. coli* K12. It can be seen that at a 2 mM concentration of levallorphan uracil incorporation is more sensitive to the inhibitory effects of the drug than is leucine incorporation. However, this difference becomes less apparent at high concentrations.

Levallorphan at a concentration of 5 mM inhibits both protein and RNA synthesis more than 90%. At this concentration RNA synthesis is completely stopped almost immediately, whereas a lag of approximately 2 min is observed before protein synthesis is completely inhibited. Figure 2 shows that at this same concentration of 5 mM the narcotic drug, levorphanol, is somewhat less potent an inhibitor of protein and RNA synthesis than

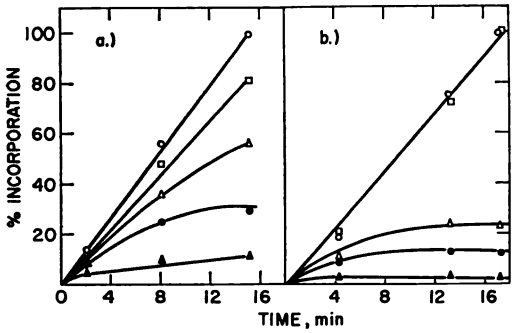



FIG. 1. *The effect of levallorphan on the incorporation of exogenous leucine-$^{14}C$ and uracil-$^{14}C$ into the trichloroacetic acid-precipitable fraction of the cells*

(a) Leucine-$^{14}C$ (0.02 $\mu$C/5 $\mu$g/ml) and levallorphan were added at $t = 0$ to an exponentially growing culture of *E. coli* K12. The following concentrations of levallorphan were used: 5 mM (▲); 3 mM (●); 2 mM (△); mM (□). The control culture (○) received no drug. The amount of incorporation of leucine-$^{14}C$ in each sample is plotted as the percentage of the 15-min control sample which was 7700 cpm/ml.

(b) Uracil-$^{14}C$ (0.05 $\mu$C/6$\mu$g/ml) was added to an identical set of cultures as in (a). The amount of incorporation of uracil-$^{14}C$ in each sample is plotted as the percentage of the 17-min control sample which was 12,500 cpm/ml.

levallorphan. Although levorphanol completely inhibits uracil incorporation within 5 min, protein synthesis continues at a slow rate for about 10 min after drug treatment. We chose to study levallorphan at a concentration of 5 mM, where the synthesis of protein and of RNA is completely inhibited, to investigate the mode of action of these drugs. At this concentration of levallorphan no loss in cell viability, as measured by colony counts on nutrient agar, is observed after 10 min of drug treatment, although 60 min of treatment does cause an approximately 25% loss of viability. Recovery from the inhibition of protein and RNA synthesis after short periods of levallorphan treatment is rapid, as can be seen in Fig. 3. The cells were treated with 5 mM levallorphan for 11 min, collected on a Millipore filter, and washed with three volumes of drug-free medium. They were then resuspended in a medium containing either leucine-$^{14}C$ or uracil-$^{14}C$

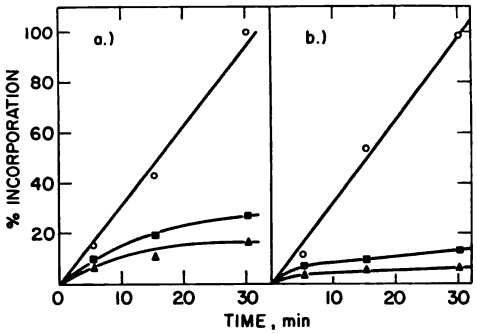



FIG. 2. *The effect of levallorphan and levorphanol on the incorporation of exogenous leucine-$^{14}C$ and uracil-$^{14}C$ into the trichloroacetic acid precipitable fraction of cells*

(a) Leucine-$^{14}C$ (0.02 $\mu$C/10 $\mu$g/ml) was added to a culture of *E. coli* K12 at the same time as 5 mM levorphanol (■) or 5 mM levallorphan (▲). The control culture (○) received no drug. The amount of incorporation of leucine-$^{14}C$ is plotted as the percentage of the 30-min control sample which was 3390 cpm/ml.

(b) Uracil-$^{14}C$ (0.02 $\mu$C/36 $\mu$g/ml) was added to an identical set of cultures as in (a). The amount of incorporation of uracil-$^{14}C$ in each sample is plotted as the percentage of the 30-min control sample which was 3230 cpm/ml.

without drug. It is apparent from Fig. 3 that after being washed free of levallorphan the cells immediately begin to synthesize protein at the same rate as the untreated culture, whereas a lag of about 10 min is observed before RNA synthesis in the treated culture reaches the rate of the control culture. The absence of a lag in the recovery of protein synthesis suggests that mRNA might be protected from decay by levallorphan treatment. It is clear that the cells can recover rapidly and com-

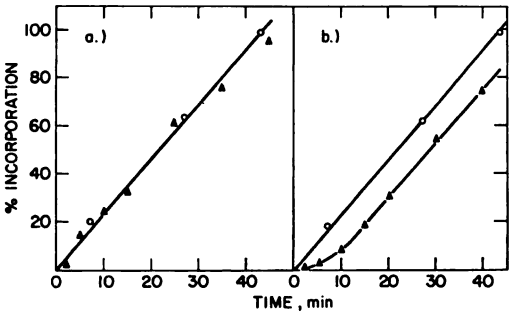



FIG. 3. *Recovery from treatment with levallorphan*

Cells were treated with 5 mM levallorphan for 11 min (▲), collected on Millipore filters, and washed three times with drug-free medium. They were then resuspended in a medium without drug containing in (a) leucine-$^{14}$C (0.02 μC/20 μg/ml) or (b) uracil-$^{14}$C (0.02 μC/45 μg/ml). The amount of incorporation of leucine-$^{14}$C or uracil-$^{14}$C is plotted as the percentage of the 45-minute control sample (○) which was 705 cpm/ml for leucine-$^{14}$C and 530 cpm/ml for uracil-$^{14}$C. The control culture was carried through filtration and washing but received no pretreatment with drug.

pletely from the effects of a 10-min treatment with levallorphan.

Since overall synthesis of RNA is strongly inhibited by levallorphan, it was of interest to see whether all forms of RNA are equally affected by drug treatment. We used sucrose gradients of crude cell extracts to determine whether any type of RNA is unaffected by levallorphan treatment. Figure 4 shows the effect of treating cells with levallorphan for 60 min in the presence of uracil-$^{14}$C. Under these conditions the uracil-$^{14}$C counts appear in the ribosomes and the soluble RNA of the control sample, but virtually no counts appear in the gradient of the drug-treated cells. Thus the synthesis of both ribosomal and soluble RNA is completely inhibited by treatment with 5 mM levallorphan.

In order to investigate the effect of levallorphan on the synthesis of mRNA, we treated the cells with levallorphan for 2 min in the presence of uracil-$^{14}$C. Figure 5 shows that in the control sample the

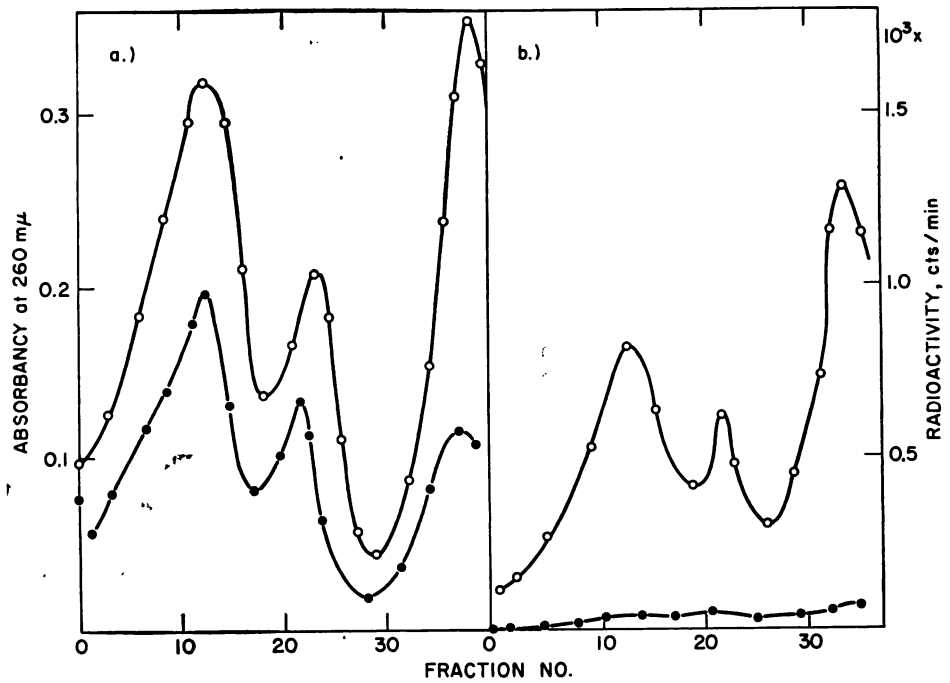



FIG. 4. *Sucrose density-gradient analysis of crude cell extracts prepared from E. coli K12 cells*

Cells were taken at (a) 60 min after uracil-$^{14}$C (0.005 μC/0.3 μg/ml) was added, and (b) 60 min after uracil-$^{14}$C (0.005 μC/0.3 μg/ml) was added in the presence of 5 mM levallorphan. Absorbancy at 260 mμ (○); trichloroacetic acid precipitable radioactive material (●).

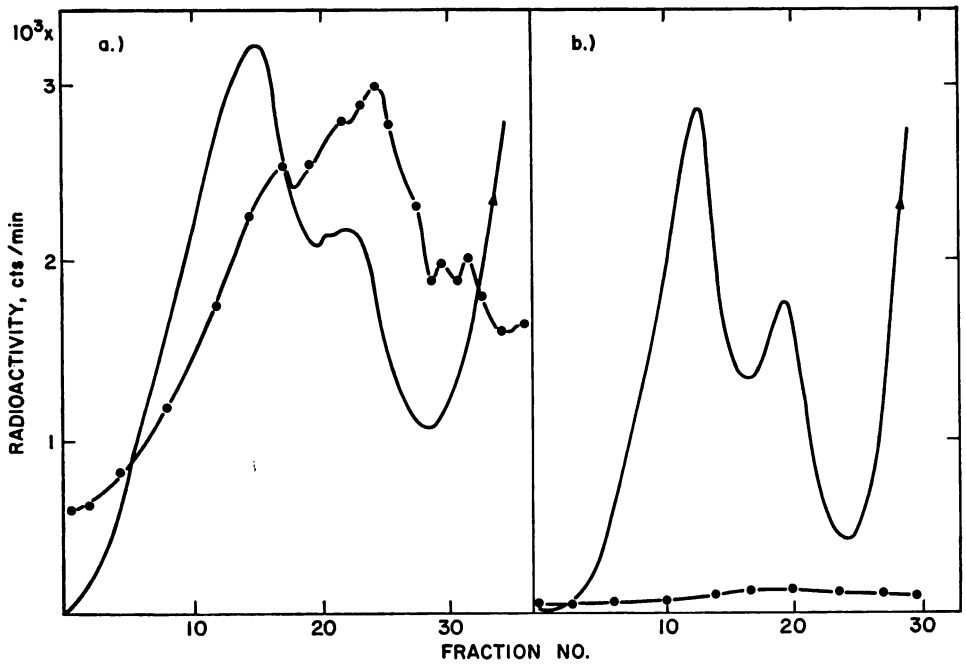



FIG. 5. *Sucrose density-gradient analysis of crude cell extracts prepared from E. coli K12*

Samples were taken at: (a) 2 min after uracil-$^{14}$C (0.02 $\mu$C/0.07 $\mu$g/ml) was added and (b) 2 min after uracil-$^{14}$C (0.02 $\mu$C/0.07 $\mu$g/ml) was added together with 5 mM levallorphan. Absorbancy at 260 m$\mu$ is a tracing of the actual graph obtained by pumping the gradient through a Gilford recording spectrophotometer. Trichloroacetic acid-precipitable radioactive material (●).

counts appear in the area generally ascribed to mRNA. In the drug-treated sample no radioactivity was incorporated into this or any other fraction. Therefore, it is clear that the synthesis of mRNA is also inhibited by levallorphan.

To determine whether the effect on mRNA synthesis is the primary effect of levallorphan, we investigated the kinetics of the inhibition of mRNA synthesis by measuring the effect of levallorphan on the induction of $\beta$-galactosidase. Cells growing on sodium succinate as the carbon source were induced with $5 \times 10^{-4}$ M IPTG and treated at various times with levallorphan. Figure 6 shows that no enzyme is formed when the drug is added together with the inducer or 2 min after the inducer. Addition of the drug 20 min after the inducer halts enzyme synthesis immediately. This result differs dramatically from that obtained with actinomycin, which is known to block the synthesis of mRNA (11). Leive (12, 13) has shown in EDTA-treated *E. coli* that actinomycin treatment results in inhibition of mRNA synthesis; however, $\beta$-galactosidase specific mRNA present before actinomycin treatment is expressed after addition of this drug. Thus, it appears that levallorphan's action is unlike that of actinomycin.

If the inhibition of RNA synthesis were the primary mode of action of levallorphan, preexisting mRNA would be expected to decay, as was found after actinomycin treatment of *E. coli* by Leive (13) and of *B. subtilis* by Levinthal *et al.* (8). To investigate this point a culture of *E. coli* K12 was exposed to uracil-$^{14}$C for 30 sec and was then treated with levallorphan. Figure 7 shows that little decrease in acid insoluble counts is observed after levallorphan treatment. Since we have already shown that levallorphan completely inhibits mRNA synthesis, the fact that preexisting mRNA does not decay after drug treatment suggested that levallorphan might actually stabilize preformed mRNA. This idea was

supported by the earlier observation that recovery of protein synthesis after washing cells free of levallorphan occurs without lag (see Fig. 3) whereas RNA synthesis does not begin until about 10 min after levallorphan has been removed. A direct test of levallorphan's ability to protect mRNA against decay was performed in

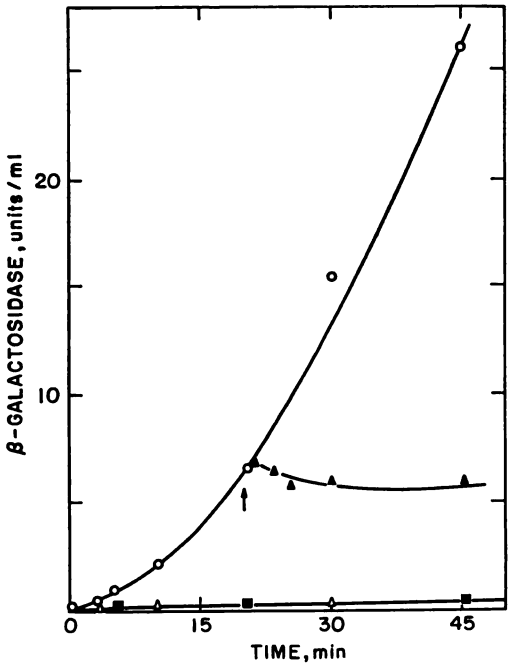



FIG. 6. *Kinetics of β-galactosidase induction*

*Escherichia coli* K12 was grown in minimal medium with sodium succinate as the carbon source. At $t = 0$, $5 \times 10^{-4}$ M IPTG was added to the culture. The culture was split, and 5 mM levallorphan was added to subcultures at 0 min (■), 2 min (△), and 20 min (▲). The control culture received no drug (○).

*B. subtilis* by utilizing the method of Fan *et al.* (14). *B. subtilis* was used in these experiments because of its sensitivity to actinomycin (8). Protein and RNA syntheses were shown to be inhibited by 5 mM levallorphan in *B. subtilis* to the same extent as in *E. coli*, i.e., more than 90%. A culture of *B. subtilis* was given a 30-second pulse of uracil-$^{14}$C and was then treated with actinomycin in the presence and absence of levallorphan. Figure 8 shows the decay curve for mRNA after actinomycin treatment. It is clear from the figure that levallorphan protects about 75% of the mRNA from decay. Treatment with levallorphan had the same effect as, in the experiments of Fan *et al.* (14), the arrest of

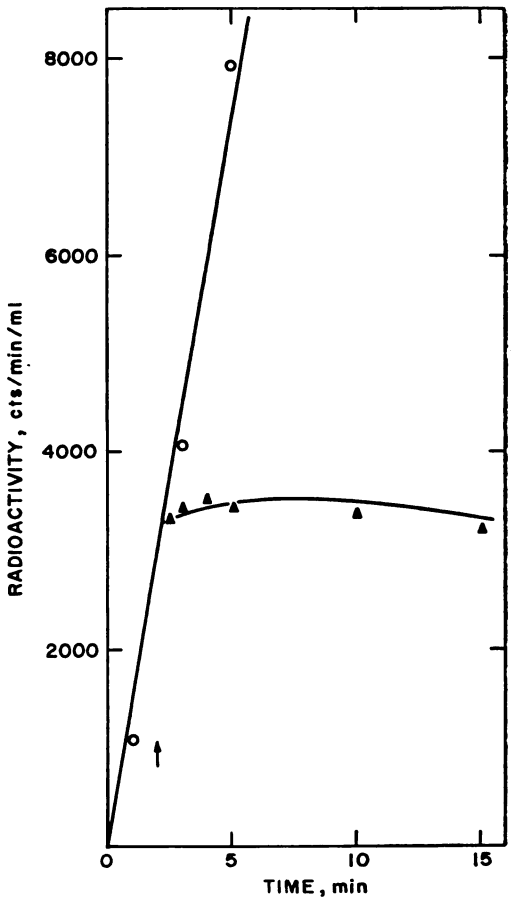



FIG. 7. *Stability of trichloroacetic acid-precipitable radioactive material after treatment with levallorphan*

Uracil-$^{14}$C (0.05 μC/0.18 μg/ml) was added to an exponentially growing culture of *E. coli* K12 at $t = 0$. At 2 min the culture was split and 5 mM levallorphan (▲) was added to half of the culture. No drug was added to the control culture (○).

energy metabolism. These authors have shown that mRNA is protected from decay when *B. subtilis*, a strict aerobe, is placed under anaerobic conditions.

Since levallorphan was shown to inhibit the synthesis of RNA and protein com-

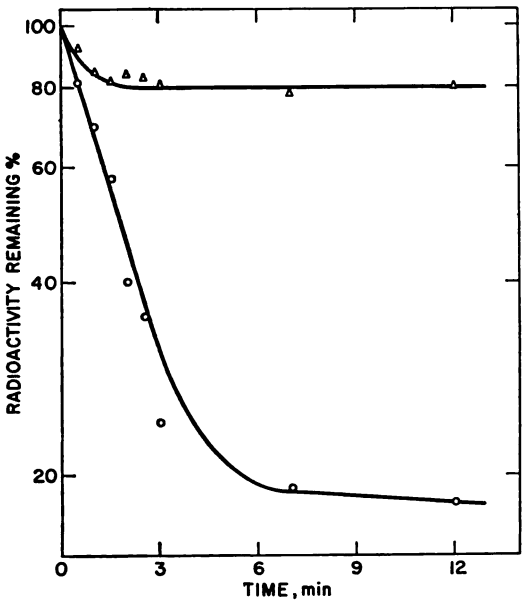



FIG. 8. *Decay of trichloroacetic acid-precipitable radioactive material in the presence of actinomycin and levallorphan in Bacillus subtilis W-23*

An exponentially growing culture of *B. subtilis* W-23 was given a 30-sec pulse of uracil-$^{14}$C (0.1 $\mu$C/0.36 $\mu$g/ml). The culture was then split; one half was treated with actinomycin 10 $\mu$g/ml (○), the other half was treated with actinomycin 10 $\mu$g/ml and 5 mM levallorphan (▲). The trichloroacetic acid-precipitable radioactive material at 30 sec gave 495 cpm/ml.

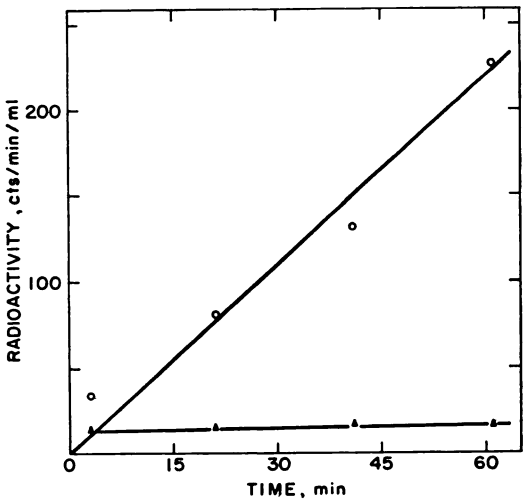



FIG. 9. *The effect of levallorphan on the incorporation of exogenous thymidine-$^{14}$C into the trichloroacetic acid-precipitable fraction of the cells*

Thymidine-$^{14}$C (0.05 $\mu$C/0.5 $\mu$g/ml) was added to an exponentially growing culture of *E. coli* K12 at $t = 0$ along with 5 mM levallorphan (▲). The control culture (○) received no drug.

pletely, it was of interest to investigate the effect of this drug on DNA synthesis. We used the incorporation of thymidine-$^{14}$C into acid-insoluble material by *E. coli* as a measure of DNA synthesis. Figure 9 shows that the synthesis of DNA is completely inhibited after 4 min of treatment with the drug. Thus levallorphan inhibits the syntheses of all three macromolecules studied.

### *The Effect of Levallorphan on ATP*

Since all macromolecular syntheses are stopped and mRNA is stabilized after treatment with 5 mM levallorphan, the effect of this drug on intracellular ATP concentration was studied. A culture of *E. coli* K12 was treated with 5 mM levallorphan. Samples of the whole culture and samples of medium filtered free of bacteria were pipetted into boiling water. These extracts were assayed for ATP as described in Materials and Methods. Figure 10 shows the effect of levallorphan on the level of ATP (curve *A*) in the whole culture, (curve *B*) in the medium, and (curve *C*) inside the cells, i.e. (*A*)-(*B*). It is apparent that after levallorphan treatment there is a rapid decrease in ATP, which is followed by a slower leakage of ATP into the medium. Thus, the inhibitory effects on the syntheses of macromolecules described above can be accounted for by the loss of intracellular ATP after levallorphan treatment.

### *The Effect of Levallorphan on Cell Permeability*

In order to determine whether the leakage of ATP into the medium observed after levallorphan treatment reflects a general effect of the drug on permeability we measured the ability of *E. coli* to take up and to concentrate galactosides in the presence of the drug. It has been shown by Koch (15) that the permease-mediated entry of

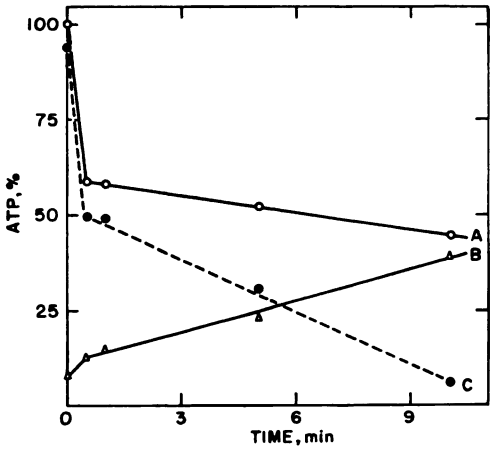



FIG. 10. *The effect of levallorphan on ATP concentration*

A culture of *E. coli* K12 was treated with levallorphan to a final concentration of 5 mM. At timed intervals samples of 1 ml of whole culture (curve *A*, ○) or 1 ml of medium filtered free of bacteria (curve *B*, △) were pipetted into 5 ml of boiling water. The ATP concentrations of these extracts were assayed using the firefly method as described in Materials and Methods. The results are plotted as the percentage of the ATP concentration of the whole culture at $t = 0$, which was $3.2 \times 10^{-4}$ M ATP. Curve *C* (●) represents the intracellular ATP concentration which is the difference between curve *A* and curve *B*.

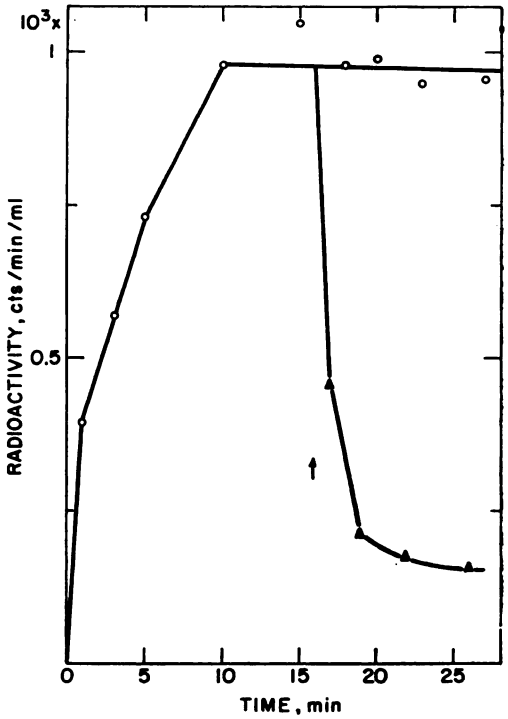



FIG. 11. *The effect of levallorphan on TMG-$^{14}$C accumulation by E. coli K12*

A preinduced culture of *E. coli* K12 growing on succinate as a carbon source was treated at −2 min with 100 μg/ml chloramphenicol to stop further protein synthesis. At $t = 0$ the culture was exposed to $^{14}$C-TMG (0.01 μC/14.7 μg/ml). After 16 minutes the culture was split: one half received 5 mM levallorphan (▲); the other half received no levallorphan (○). Samples of 1 ml were pipetted onto Millipore filters (0.45 μ) and rapidly washed with 3 volumes of cold medium containing no drug.

galactosides into the cell is not dependent on energy metabolism, whereas the active concentration of galactosides within the cell is energy dependent. Since levallorphan treatment causes a loss of intracellular ATP, it was predicted that treatment with this drug should cause accumulated galactosides to leak out of the cell. To test this prediction *E. coli* K12 growing on succinate as a carbon source was induced with $5 \times 10^{-4}$ M IPTG, allowed to accumulate the nonmetabolizable galactoside, TMG-$^{14}$C, and was then treated with levallorphan. The amount of radioactive TMG within the cell was measured by collecting the cells on a Millipore filter and counting the filters in a gas flow counter. Figure 11 shows that, as predicted, immediately after levallorphan treatment the accumulated TMG is lost.

The rate of entry of metabolizable galactosides such as ONPG should not be affected by levallorphan treatment unless this drug alters the permeability of the cell. To investigate this a culture of *E. coli* K12 containing an inducible permease for lactose was grown on succinate as a carbon source and induced with $5 \times 10^{-4}$ M IPTG. The intact cells were then exposed to 4 mM ONPG and the rate of *o*-nitrophenol production was measured spectrophotometrically. Figure 12 shows that the rates of hydrolysis of ONPG by whole, permease-containing cells are virtually identical in the presence and absence of levallorphan. As a further demonstration that levallor-

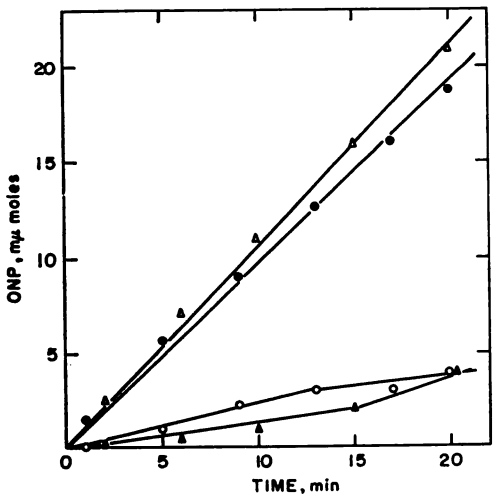



FIG. 12. *The effect of levallorphan on the rate of ONPG hydrolysis by whole cells*

A preinduced culture of *E. coli* ($i^+$, $z^+$, $y^+$) was first treated with 100 μg/ml of chloramphenicol to stop further β-galactosidase synthesis and then exposed to 4 mM ONPG in the presence (●) and absence (△) of 5 mM levallorphan. The rate of ONP production by whole cells was measured spectrophotometrically. The same experiment was performed in *E. coli* AB1105 ($i^+$, $z^+$, $y^-$) in the presence (▲) and absence (○) of 5 mM levallorphan.

phan does not have a general effect on permeability a similar experiment was done using *E. coli* AB1105, a mutant lacking the permease for lactose. Again cells growing on succinate were induced with IPTG and then exposed to ONPG in the presence of levallorphan. It is apparent from Fig. 12 that the entry of ONPG into this permeaseless strain is not enhanced by treatment with levallorphan. Thus, it is clear that levallorphan neither increases nor decreases the rate of entry of galactosides into permease positive or negative cells, although it readily stops the cells from concentrating TMG-$^{14}$C.

We also investigated the effect of levallorphan treatment on the permeability of the cells to macromolecules. The cell proteins were prelabeled with leucine-$^{14}$C for 30 min. The culture was then treated with levallorphan in the presence of cold leucine. Samples of the whole culture and the medium were assayed for acid-precipitable counts. Table 1 clearly shows that levallorphan does not cause proteins to leak out into the medium.

It appears therefore that the leakage of ATP into the medium following treatment with levallorphan does not reflect a general change in the permeability of the cell membrane.

### *The Effect of Levallorphan on ATP Synthesis*

A possible cause of the rapid decrease in intracellular ATP concentration seen after levallorphan treatment might be the arrest of ATP synthesis. This might occur through interference with respiration or oxidative phoshorylation.

The effect of levallorphan on the respiration of *E. coli* was studied using a Warburg apparatus. Figure 13 compares cells

TABLE 1

*The absence of leakage of protein in cells treated with levallorphan*

A culture of *Escherichia coli* K12 was prelabeled with leucine-$^{14}$C (0.02 μC/10 μg/ml) for 30 min. After 30 min a 100-fold excess of cold leucine was added and the culture was split. Half was treated with 5 mM levallorphan; the other half received no drug. The radioactivity of the trichloroacetic acid-precipitable material in both the whole culture and the medium filtered free of cells was determined.

| Time after drug addition (min) | Control | | Levallorphan 5 mM | |
|---|---|---|---|---|
| | Cells + medium | Medium | Cells + medium | Medium |
| 0 | 4060[a] | 460 | 4060 | 460 |
| 5 | — | 450 | — | 320 |
| 10 | 4950 | 494 | 4260 | 300 |

[a] Values are expressed as counts per minute per milliliter.

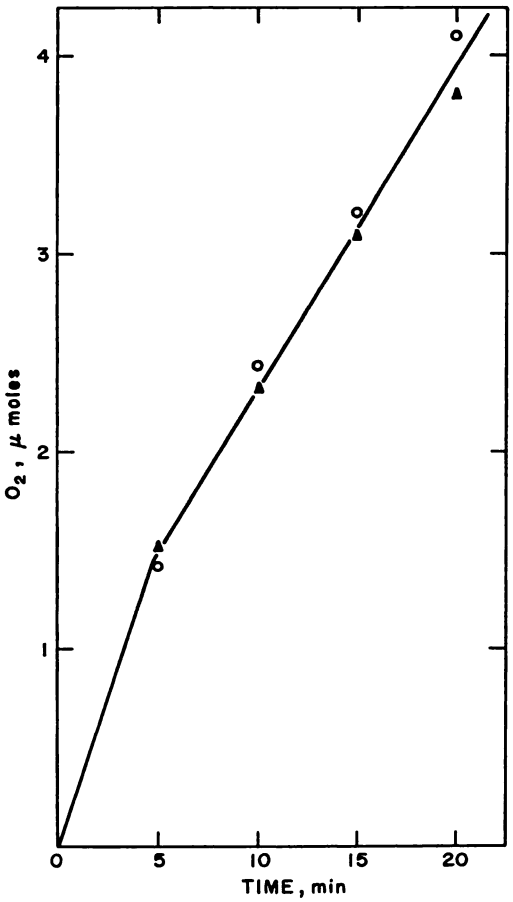



FIG. 13. *The effect of levallorphan on oxygen consumption*

Samples of a culture of *E. coli* K12 growing exponentially on glucose were placed in Warburg vessels at 37°. At $t = 0$ 5 mM levallorphan was added to one culture from the side arm of the vessel (▲). At the same time 100 μg/ml of chloramphenicol was added to the control culture (○). The amount of oxygen consumed was measured directly from the manometer.

treated with levallorphan with cells whose growth is arrested by chloramphenicol. It is apparent that the oxygen uptake is the same in both cases. Similarly, as shown in Fig. 14 the rate of disappearance of glucose from the culture is unaffected by levallorphan treatment. It is therefore clear that the decrease in ATP concentration seen after levallorphan treatment is not due to the drug's interference with respiration.

That levallorphan does not produce its effect on ATP concentration by uncoupling oxidative phosphorylation is apparent from the fact that the drug is an effective inhibitor of macromolecular syntheses in a condition where oxidative phosphorylation

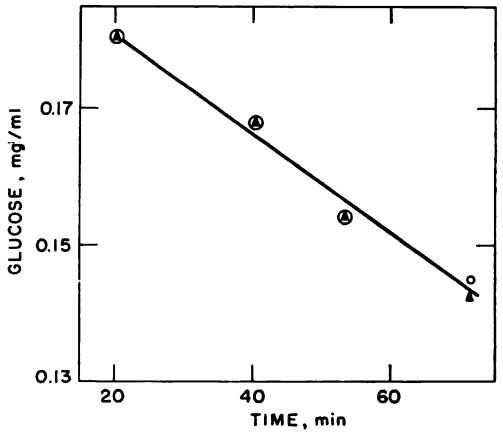



FIG. 14. *The effect of levallorphan on glucose metabolism*

A culture of *E. coli* K12 growing on glucose as a carbon source was treated at $t = 0$ with 5 mM levallorphan (▲). Timed samples of the whole culture were assayed for glucose by the glucostat method (see Materials and Methods). The control culture (○) received 100 μg/ml of chloramphenicol to stop growth.

does not take place. Figure 15 shows that in *E. coli* K12 which had been adapted to anaerobic growth on glucose for many generations levallorphan promptly arrests protein and RNA synthesis in the absence of oxygen.

Since levallorphan does not appear to inhibit respiration or oxidative phosphorylation we investigated the fate of ATP after drug treatment in greater detail. *E. coli* K12 growing on minimal medium with glucose as a carbon source was prelabeled for one generation with adenine-$^{14}C$ and treated with 5 mM levallorphan. After treatment, the cells and medium were separated by filtration and each was extracted and chromatographed as described. Figure 16 shows the actual peaks obtained by counting the chromatograms in a Nuclear Chicago strip counter. It is apparent from the figure that most of the label in the con-

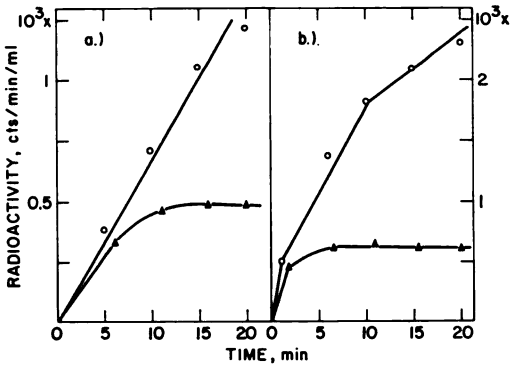



FIG. 15. *The effect of levallorphan on the incorporation of exogenous leucine-$^{14}$C and uracil-$^{14}$C into the trichloroacetic acid-precipitable fraction of cells growing anaerobically on glucose*

(a) At $t = 0$ leucine-$^{14}$C (0.03 $\mu$C/1 $\mu$g/ml) was added to a culture of *E. coli* K12 which had been growing anaerobically (under argon and $CO_2$) for many generations. Levallorphan (▲) was added to a final concentration of 5 mM at the same time as the leucine-$^{14}$C. An identical culture without drug served as a control (○).

(b) At $t = 0$ uracil-$^{14}$C (0.01 $\mu$C/5 $\mu$g/ml) and 5 mM levallorphan (▲) were added to *E. coli* K12 growing anaerobically. An identical control culture received no drug (○).

trol cells is in the form of ATP and that no label is found in the control medium. In contrast, after 10 min of levallorphan treatment almost no ATP is seen inside the cell. An increase of intracellular ADP and AMP is observed in the treated cells, as well as leakage into the medium of nucleotides mostly in the form of AMP. Table 2 presents the same as well as additional data in a different form. The radioactive peaks were cut out and weighed. An arbitrary value of 100 was assigned to the intracellular ATP peak of the control culture. All other peaks were assigned a value relative to this ATP peak. It can be seen from Table 2 that nucleotides continue to leak from the cell after 10 min of levallorphan treatment. By 20 min almost no adenine nucleotides remain inside the cell and most of the label can be accounted for in AMP and ADP found in the medium. Similar results are shown in Table 2 for treatment of cells with the narcotic analog of levallorphan, levorphanol. In contrast to these results, the table shows that treatment of cells with chloramphenicol neither decreases the ATP concentration in the cell nor causes the leakage of nucleotides into the medium. Thus, the effect on nucleotides after levallorphan and levorphanol treatment is not merely a reflection of the arrest of protein synthesis.

Since experiments described earlier had suggested that levallorphan does not interfere with respiration or oxidative phosphorylation, we compared the adenine nucleotide levels in a levallorphan-treated culture with those of a culture whose ATP synthesis was limited by sodium azide. Table 2 shows that sodium azide treatment causes neither the ATP concentration to decrease nor nucleotides to leak from the cell. However, since azide may have other effects than the arrest of ATP synthesis, another method of stopping ATP production was used.

*Escherichia coli* K12 was grown on minimal medium with succinate as the sole carbon source. Under these conditions ATP production can occur only through oxidative pathways. The culture was prelabeled with adenine-$^{14}$C for one generation and was then filtered free of label. It was divided into two parts; one was treated with 5 mM levallorphan for 10 min, the other was placed under an anaerobic condition. It can be seen from Table 3 that the effect of levallorphan treatment on succinate grown cells is similar to that on glucose grown cells shown in Table 2. After 10 minutes of drug treatment no ATP remains inside the cells. Most of the original adenine-$^{14}$C label is found in the medium as adenosine or adenine rather than as AMP, which is the case with cells growing on glucose. Surprisingly, in contrast to these results the culture whose ATP synthesis is stopped for 10 min by being deprived of oxygen shows almost no change in ATP concentration and no leakage of nucleotides into the medium. Nonetheless, Fig. 17 demonstrates that placing cells growing on succinate acutely into anaerobiosis immediately arrests the synthesis of both RNA and protein. Stopping ATP synthesis thus appears quite different than

TABLE 2

*The effect of inhibitors on adenine nucleotides of E. coli K12 during aerobic growth on glucose*

Cultures of *E. coli* K12 were prelabeled with adenine-$^{14}$C (10 $\mu$C/21.6 $\mu$g/ml) for one generation, filtered free of label, and resuspended in medium without radioactive adenine. They were then treated as indicated in the table, after which the cells and medium were separated by filtration and the nucleotides of each fraction were extracted and separated as described in Materials and Methods. The thin-layer chromatograms were counted in a strip counter as shown in Fig. 16. The radioactive peaks obtained were then cut out and weighed. The intracellular ATP peak of the control culture was assigned an arbitrary value of 100, and all other peaks were assigned a value relative to this peak. Experiments 1 and 2 in this table are the same experiments shown in Fig. 16.

| Experiment | Treatment | ATP | ADP | AMP | Adenosine or adenine |
|---|---|---|---|---|---|
| 1. Cells | None | 100 | 13 | 24 | 13 |
| Medium | | 10 | 0 | 0 | 0 |
| 2. Cells | 5 mM levallorphan, 10 min | 0 | 19 | 48 | 0 |
| Medium | | 20 | 0 | 72 | 0 |
| 3. Cells | 5 mM levallorphan, 20 min | 0 | 0 | 10 | 0 |
| Medium | | 8 | 30 | 132 | 0 |
| 4. Cells | 5 mM levorphanol, 10 min | 5 | 33 | 25 | 0 |
| Medium | | 26 | 25 | 75 | 0 |
| 5. Cells | 100 $\mu$g/ml chloramphenicol, 10 min | 88 | 38 | 27 | 0 |
| Medium | | 4 | 0 | 0 | 0 |
| 6. Cells | 10 mM Na azide, 10 min | 90 | 37 | 32 | 0 |
| Medium | | 0 | 0 | 0 | 0 |

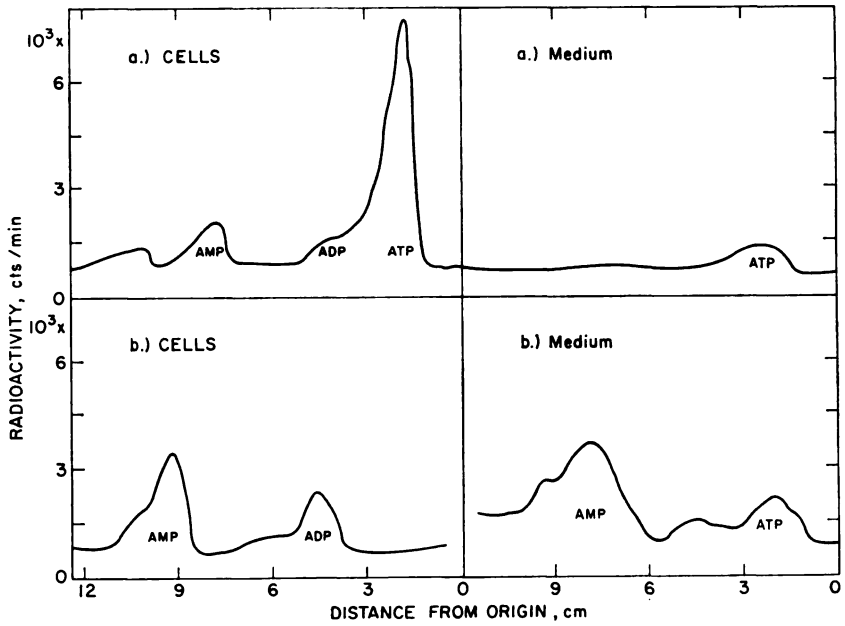



FIG. 16. *The effect of levallorphan on adenine nucleotides*

A culture of *E. coli* K12 growing exponentially on glucose as a major carbon source was prelabeled with adenine-$^{14}$C (10 $\mu$C/21.6 $\mu$g/ml) for one generation, filtered free of label, and resuspended in medium without radioactive adenine. The culture was split and placed on a New Brunswick shaker at 37° for 10 min in the presence (b) and absence (a) of 5 mM levallorphan. At 10 min the cells and medium were separated by filtration and the nucleotides of each fraction were extracted and separated on TLC as described in Materials and Methods. The graphs obtained by counting the thin-layer chromatograms in a strip counter are shown in the figure. The radioactive peaks were identified by the UV absorption of known nucleotides run on each chromatogram.

TABLE 3

*The effect of levallorphan on adenine nucleotides of E. coli K12 growing on succinate*

Cultures of *E. coli* K12 growing on succinate as the sole carbon source were labeled with adenine-$^{14}$C as described in Table 2.

| Experiment | Treatment | ATP | ADP | AMP | Adenosine or adenine |
|---|---|---|---|---|---|
| 1. Cells | None | 100 | 41 | 10 | 0 |
| Medium | | 0 | 0 | 0 | 0 |
| 2. Cells | 5 mM levallorphan, 10 min | 0 | 16 | 16 | 0 |
| Medium | | 0 | 0 | 0 | 82 |
| 3. Cells | Argon $CO_2$, 10 min | 105 | 47 | 18 | 0 |
| Medium | | 0 | 0 | 0 | 0 |
| 4. Cells | Argon $CO_2$, 10 min + 5 mM levallorphan | 102 | 31 | 22 | 0 |
| Medium | | 0 | 0 | 0 | 0 |

levallorphan treatment with respect to the changes seen in the nucleotide pools.

It was of interest to investigate the effect of stopping ATP synthesis in the presence of levallorphan. Prelabeled, succinate-grown cells were placed in an anaeroic condition at the same time as levallorphan treatment was begun. Table 3 shows that

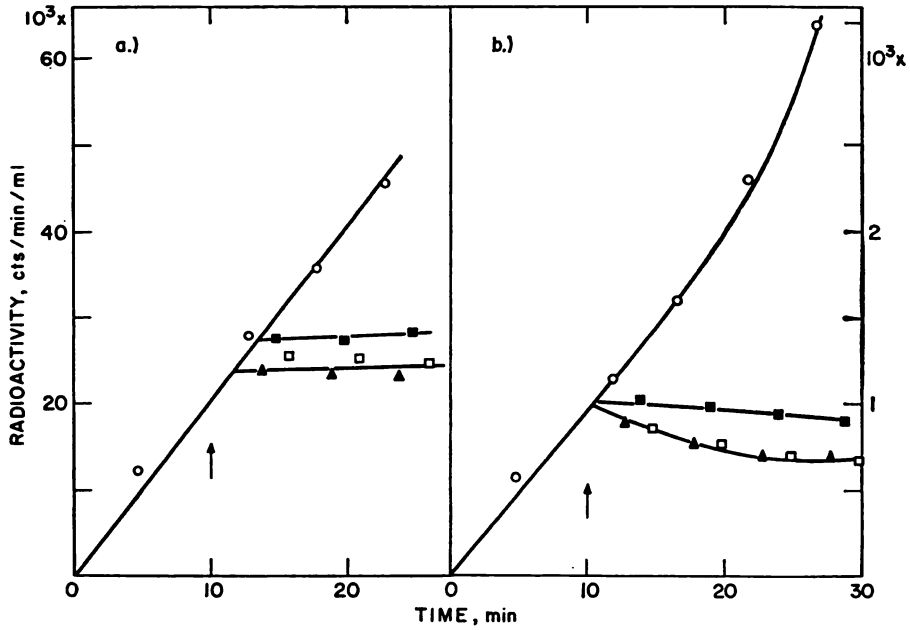



FIG. 17. *The effect of acute anaerobiosis on the incorporation of exogenous leucine-$^{14}$C and uracil-$^{14}$C into the trichloroacetic acid-precipitable fraction of E. coli K12 growing on succinate as a carbon source.*

(a) Leucine-$^{14}$C (0.1 $\mu$C/5 $\mu$g/ml) was added at $t = 0$ to a culture of *E. coli* K12 growing exponentially on succinate as a major carbon source. At 10 min the culture was split and either treated with 5 mM levallorphan (▲); placed in an oxygen-free atmosphere, i.e., argon $CO_2$ (■); or placed in an oxygen-free atmosphere in the presence of 5 mM levallorphan (□). The control culture was continually aerated and received no drug (○).

(b) Uracil-$^{14}$C (0.04 $\mu$C/4.8 $\mu$g/ml) was added to a culture of *E. coli* K12 growing aerobically on succinate as in (a). At 10 min the culture was split and treated as described in (a).

TABLE 4

*The effect of levallorphan on adenine nucleotides of E. coli K12 growing anaerobically on glucose*

Cultures of *E. coli* K12 growing anaerobically on glucose were labeled with adenine-$^{14}$C as described in Table 2.

| Experiment | Treatment | ATP | ADP | AMP | Adenosine or adenine |
|---|---|---|---|---|---|
| 1. Cells | None | 100 | 0 | 0 | 0 |
| Medium | | 0 | 0 | 17 | 0 |
| 2. Cells | 5 mM levallorphan, 10 min | 0 | 0 | 11 | 0 |
| Medium | | 22 | 58 | 22 | 0 |

the drug does not cause any decrease in ATP concentration under these conditions.

That anaerobiosis per se is not sufficient to stop drug action is seen from Fig. 15 and Table 4. A culture of *E. coli* K12 adapted to growth on glucose under anaerobiosis for many generations was prelabeled for one generation with adenine-$^{14}$C prior to treatment with levallorphan. It is apparent from Table 4 that the effect of levallorphan treatment on cells growing anaerobically on glucose is similar to that found for cells growing aerobically on glucose, i.e, intracellular ATP is destroyed and nucleotides leak into the medium.

The effect of levallorphan treatment on guanine nucleotides was also studied. *E. coli* K12 growing aerobically on glucose was prelabeled with guanine-$^{14}$C in the presence of a 5-fold excess of cold adenine to minimize the conversion of guanine to adenine nucleotides. Cells were treated with 5 mM levallorphan for 10 min, and the nucleotides were extracted and separated as described above. Table 5 shows that the effect of levallorphan on guanine nucleotides is similar to that on adenine nucleotides. The GTP concentration within the cell decreases after 10 min of drug treatment, and leakage of GMP and GDP is observed. These results could either reflect an event secondary to ATP loss or the destruction of all of the nucleoside triphosphates.

### *The Effect of Levallorphan on HeLa Cells*

Levallorphan, an analog of morphine, causes a dramatic decrease in intracellular triphosphate concentrations and a cessation of macromolecular syntheses in bacteria. Because of the obvious pharmacologic significance of narcotic compounds, it was of interest to see whether levallorphan action on mammalian cells was similar. Therefore, we studied the effects of levallorphan on HeLa cells. Figure 18 shows the effect of various concentrations of levallorphan on leucine-$^{14}$C incorporation by HeLa cells. Cells were exposed to leucine-$^{14}$C at time 0 and the drug was added 5 min later. It is apparent from Fig. 18 that protein synthesis is essentially stopped within 5 min of adding 3 mM levallorphan. At this concentration of drug

TABLE 5

*The effect of levallorphan on guanine nucleotides in E. coli K12*

Cultures of *E. coli* growing on glucose as a carbon source were prelabeled with guanine-$^{14}$C (10 $\mu$C/21 $\mu$g/ml) for one generation and then treated as described in Table 2. The intracellular GTP peak of the control culture was assigned a value of 100, and all other peaks were assigned a value relative to this peak.

| Experiment | Treatment | GTP | GDP | GMP |
|---|---|---|---|---|
| 1. Cells | None | 100 | 34 | 0 |
| Medium | | 7 | 7 | 0 |
| 2. Cells | 5 mM levallorphan, 10 min | 34 | 38 | 0 |
| Medium | | 7 | 84 | 0 |

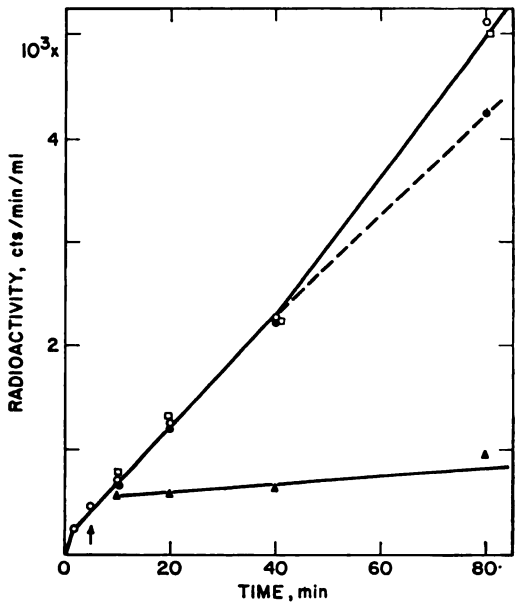



FIG. 18. *The effect of levallorphan on the incorporation of exogenous leucine-$^{14}$C into the trichloroacetic acid-precipitable fraction of HeLa cells*

HeLa cells growing in spinner culture were exposed to leucine-$^{14}$C (0.3 $\mu$C/1 $\mu$g/ml) at $t = 0$. At 5 min the culture was split and treated with the following concentrations of levallorphan: $10^{-3}$ M (□); $10^{-4}$ M (●); or $3 \times 10^{-4}$ M (▲). The control culture received no drug (○).

RNA synthesis is also stopped as can be seen in Fig. 19. In this experiment HeLa cells were exposed to leucine-$^{14}$C or uridine-$^{14}$C at the same time as drug treatment was begun. Essentially no incorporation of either labeled compound is observed in treated cells.

The effect of levallorphan on the nucleotides of HeLa cells was studied in an experiment similar to those described above with bacteria. Adenine nucleotides were labeled with adenosine-$^{14}$C for 15 min. The cells were then washed free of the label and resuspended for 10 min in medium containing 3 mM levallorphan. Cellular nucleotides were extracted with perchloric acid in a similar manner to that described above for bacteria, separated by TLC and counted in a strip counter. Table 6 shows the results of such an experiment. Treatment of HeLa cells with 3 mM levallorphan causes a loss of about 60% of the intracellular ATP. A concomitant increase in intracellular AMP and ADP is observed which nearly accounts for the loss of label from ATP. Although the destruction of ATP is not as complete in HeLa cells as in bacteria, it is of sufficient magnitude to suggest that in HeLa cells levallorphan produces its inhibition of RNA and protein synthesis as a result of its effect on ATP concentration.

## DISCUSSION

The results presented in the preceding section show that treatment of *E. coli* with 5 mM levallorphan promptly arrests the synthesis of protein, DNA, and all classes of RNA. The fact that upon treatment with the drug the intracellular ATP is rapidly converted to ADP and AMP which, together with a remnant of ATP, leak into the medium accounts well for the arrest of the synthesis of macromolecules, since in the absence of ATP energy-requiring reactions cannot proceed.

The cause of the disappearance of ATP is not obvious; either the inhibition of ATP formation or the acceleration of ATP hydrolysis by the drug could be responsible. We investigated, therefore, the effect of sodium azide, a drug known to interfere with ATP synthesis, on the intracellular level of ATP. We found that this poison arrests the synthesis of macromolecules, but causes very little change in the intracellular ATP level (Table 2). Similarly, oxygen deprivation in cells using succinate as the sole source of energy, promptly arrested the synthesis of macromolecules, but failed to cause a significant reduction in the level of ATP (Fig. 17, Table 3). It would appear, therefore, that the arrest of ATP production neither reduces the level of ATP significantly, nor causes leakage of nucleotides into the medium. However, the evidence for this view is not completely convincing. For example, sodium azide might have other effects than inhibition of ATP synthesis: it might reduce the rate of formation of precursors of protein and nucleic acids. Similarly, depriving cells growing on succinate of oxygen, may prevent the syn-

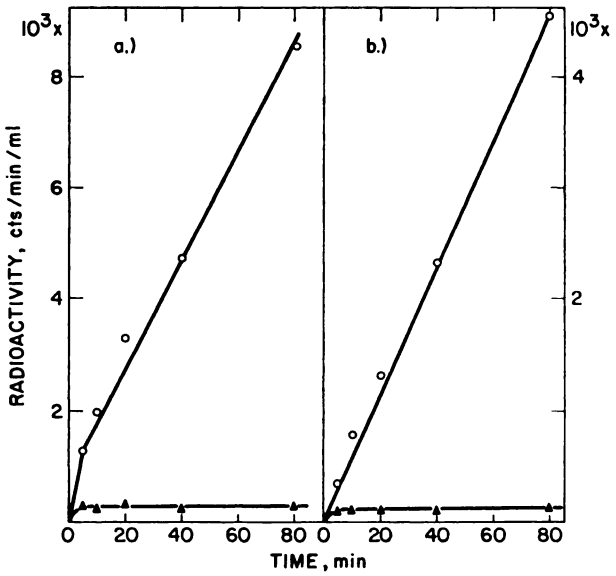



FIG. 19. *The effect of levallorphan on the incorporation of exogenous leucine-$^{14}$C and uridine-$^{14}$C of HeLa cells*

(a) Leucine-$^{14}$C (0.16 $\mu$C/0.5 $\mu$g/ml) was added to a spinner culture of HeLa cells at the same time as 3 mM levallorphan (▲). The control culture received no drug (○).

(b) Uridine-$^{14}$C (0.03 $\mu$C/0.29 $\mu$g/ml) was added to an identical set of cultures as in (a).

thesis of such precursors from succinate. In that case, the synthesis of macromolecules could only proceed at the cost of the amino acids and nucleotides already present in the intracellular pools and might come to a halt due to the lack of a required building block, before a significant portion of the intracellular ATP has been utilized. We did not investigate the effect of supplying the oxygen-deprived cells with a mixture of the amino acids required for protein synthesis and nucleosides, since components of this mixture, such as serine and adenosine, can serve as sources of energy under anaerobic conditions.

The observation, shown in Table 3, that levallorphan does not produce a drop in the ATP level of succinate-grown cells deprived of oxygen would be in good agreement with the concept that the drug arrests synthesis of ATP, but that in these cells the lack of building blocks prevents the exhaustion of the ATP.

However, it is difficult to see at what stage of ATP synthesis levallorphan might exert its inhibitory effect. The drug causes

TABLE 6

*The effect of levallorphan on adenine nucleotides in HeLa cells*

A spinner culture of HeLa cells was prelabeled with adenosine-$^{14}$C (10 $\mu$C/25 $\mu$g/ml) for 15 min, collected by centrifugation and resuspended in medium without labeled adenosine for 10 min in the presence and absence of 3 mM levallorphan. The cells were then collected by centrifugation and their nucleotides were extracted and separated as described in Materials and Methods. The peaks obtained by counting the chromatograms were cut out and weighed. An arbitrary value of 100 was assigned to the control peak of ATP; all other peaks were assigned relative values according to their weight.

| Experiment | Treatment | ATP | ADP | AMP |
|---|---|---|---|---|
| 1. Cells | None | 100 | 17 | 0 |
| 2. Cells | 3 mM levallorphan, 10 min | 41 | 26 | 12 |

a loss of intracellular ATP in cells growing on glucose in the presence, as well as the absence of oxygen (Table 4, Figs. 15 and 16); consequently, levallorphan cannot be considered to act by uncoupling oxidative phosphorylation. Moreover, the drug-treated cells metabolize glucose as fast and with the same uptake of oxygen as untreated cells (Figs. 13 and 14); consequently levallorphan cannot be considered to act by inhibiting the energy-producing reactions.

The failure to demonstrate an effect of levallorphan on energy production, suggests that the drug acts by stimulating the hydrolysis of ATP. We could not demonstrate a stimulation of the already high ATPase activity of cell extracts by levallorphan. However, this failure may reflect the fact that the destruction of the cell membrane activates the very ATPase we wish to study. The failure of levallorphan to cause a reduction of the ATP level when added to cells deprived of oxygen in a medium containing succinate as source of energy, appears to contradict a direct stimulation of ATP hydrolysis by the drug. However, this finding may reflect the inability of levallorphan to enter a cell whose metabolism has been arrested or the fact that the drug has to be chemically altered by oxidation or dehydrogenation to become effective.

On the balance, we favor the view that activation of an ATPase is the mode of action of levallorphan. Although not studied in great detail, Fig. 2 and Table 2 indicate that similar conclusions can be made for levorphanol, the narcotic analog of levallorphan.

The leakage of nucleotides into the medium observed after levallorphan treatment could either be secondary to the loss of intracellular ATP or a direct effect of the drug on cell permeability. Figure 12 shows that levallorphan treatment does not change the rate of entry of galactosides into *E. coli* whereas it does stop the accumulation of TMG-$^{14}$C (Fig. 11). These results are consistent with an effect on ATP concentration, and not a change in cell permeability. In addition, the time course of leakage of nucleotides is such that AMP and ADP first increase in concentration inside the cell before they completely leak out (Table 2). This is consistent with the hypothesis that ATP is converted to ADP and AMP inside the cell, and as a result of the loss of ATP the nucleotides can no longer be held within the cell against a concentration gradient.

Our results and conclusions on the mode of action of levallorphan and levorphanol differ from those of Simon *et al.* (2, 3). Using lower drug concentrations (1–2 mM) these authors have presented evidence that levorphanol is a selective inhibitor of ribosomal RNA synthesis. They have reported that at low levels of levorphanol DNA, protein, mRNA, and transfer RNA continue to be synthesized, but ribosomal RNA is not made (3). In view of our studies showing such a dramatic decrease in intracellular ATP concentration after treatment with 5 mM levallorphan and levorphanol, it is not unreasonable to assume that the low concentrations of drug used by Simon could produce the situation of "shift down" (16). In other such situations wherein a culture is shifted from good to relatively poor growth conditions it has been shown that ribosomal RNA synthesis is selectively inhibited (17). Thus, the selectivity reported by Simon probably reflects the "shift down" conditions caused by marginal concentrations of the drug. The fact that Simon *et al.* (18) have also reported selective inhibition of ribosomal RNA synthesis with dinitrophenol, a known uncoupling agent, supports the contention that they are observing the results of "shift down" in the case of both dinitrophenol and levorphanol.

We have shown that levallorphan and levorphanol produce both a strong inhibition of macromolecular syntheses and a decrease in intracellular ATP concentration in bacteria. In a recent paper Cousin *et al.* (19) have reported a remarkably similar situation in the thermosensitive mutant *E. coli* CR 341-T28. Although this mutant will grow well at 30°, it does not make RNA, DNA, or protein at 40°. These authors have shown that the level of ATP in

these cells decreases dramatically when they are placed at 40°. Although this mutant has not been studied extensively, it may prove to be a physiological example of the same effect caused by levallorphan and levorphanol.

Because of the importance of narcotics in clinical medicine it was of interest to see whether the same results found in bacteria were to be obtained also in mammalian cells. We have shown that levallorphan acts in HeLa cells (Figs. 18 and 19 and Table 6) in a similar way as in bacteria. In a recent paper Noteboom and Mueller (20) also present evidence that levallorphan inhibits RNA and protein synthesis in HeLa cells. Moreover, using extracts of HeLa cells they were unable to show any effect of levallorphan on the *in vitro* synthesis of RNA or protein. We also could not show inhibition by levallorphan of protein synthesis *in vitro* using *E. coli* extracts. Noteboom's results are consistent with the suggestion that levallorphan's primary action in HeLa cells is the destruction of ATP.

The decrease in ATP that we observed after treating HeLa cells with levallorphan may be related to its mechanism of action in producing analgesia in humans (Arthur S. Keats, personal communication). Although the concentration of drug needed in the present studies are far higher than those needed to produce analgesia, it is possible that selective neurons are more sensitive to the drug than are HeLa cells.

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