it feasible to trace very small fractions of ingested PSP. In the first experiment the lack of radioactivity in urine was the only evidence for non-absorption. However, it was considered that the absence of radioactive material in urine would not prove that ingested radioactivity had not been absorbed since absorbed material might be accumulated in the body or be metabolized and excreted *via* the biliary-fecal route. Therefore, analysis of blood samples drawn at 2 different times after ingestion of the radioactive meal was included in the second experiment. It seems very probable that lack of radioactivity in both blood and urine indicates little or no absorption of the labeled material. Quantification of radioactivity in feces was complicated by the difficulty in obtaining homogeneous samples and by the introduction of color into the counting fluid. In the first experiment about 87% of ingested radioactivity was accounted for in the feces of one human subject. In the second experiment the use of C^{14}-labeled PSP and liquid scintillation counting allowed the analyses of radioactivity in feces to be more accurate. The recovery in the feces of 3 subjects of 93-101% of ingested radioactivity lends strong support to the conclusion that little radioactivity had been absorbed. An enterohepatic circulation of labeled material may have occurred, but the lack of detectable radioactivity in either blood or urine argues against this hypothesis.

Summary. Polydiethylstilbestrol phosphate (PSP), an effective growth promoting agent in beef cattle, tends to accumulate in the animal's liver as indicated by studies in which radioactive PSP was used. Upon ingestion of such liver (cooked) by human beings, 93-101% of the radioactivity was found in the feces. No radioactivity could be detected in either blood or urine. It is concluded that neither PSP nor its metabolites ingested under such circumstances are absorbed in detectable amounts during passage through the human intestinal tract.

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Lysine Inhibition of Cell Protein and Viral Synthesis. Reversal by Other Amino Acids.* (30360)

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In virus-infected cells maintained in a minimal medium, addition of an excess of amino acids such as lysine, tryptophane or phenylalanine causes viral inhibition which is reversible by addition of certain other amino acids (1,2). This effect of lysine on replication of influenza virus hemagglutinins was of special interest because a wide variety of amino acids also reversed the moderate inhibition of cell protein synthesis. Krebs 2 ascites tumor cells infected with a high multiplicity of influenza strain PR_8 which give good yields of cell-associated viral hemagglutinins and CF antigen but no additional infectious virus(3) have several advantages as an experimental system. One-step replication and accurate measurement of viral structural protein as the titer of unassembled intracellular hemagglutinins facilitates interpretation of results. In addition ascites tumor cells have been found

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by a number of investigators to be suitable for studies on biosynthesis of macromolecules and membrane transport of amino acids. The present paper reports the results of experiments to evaluate the role of lysine transport in the observed inhibition and reversal of cell protein synthesis and viral HA formation and a preliminary search for combined excess lysine-C^{14} in the protein-nucleic acid fraction of the cells.

Materials and methods. Krebs 2 ascites tumor cells were infected with the PR_8 strain of influenza virus at a multiplicity of approximately 10 EID_{50} per cell. Experimental procedures and basic medium containing glutamate and glucose as the only organic constituents have been described(1,4). Amino acids were reagent grade or analyzed by chromatography. Generally the natural l-isomers were used but some experiments were done with synthetic dl- or d-isomers.

To measure cellular protein synthesis leucine-l-C^{14} or phenylalanine C^{14} (uniformly labeled) were added to uninfected cell suspensions at a concentration of 10^4 CPM/ml (1 CPM/100 cells), unless otherwise noted, with 2.5 μg/ml of carrier amino acid. No differences were noted between leucine labeled on COOH and uniformly labeled leucine. Uptake of C^{14} label into the cold trichloracetic acid precipitable fraction of Krebs 2 cells was measured by a standard method(1). Some determinations were also done with lysine C^{14} (ul) mixed with cold lysine to give a constant specific activity and added at various concentrations.

Measurements of lysine in the free amino acid pool were done by extracting washed Krebs 2 cells in boiling water according to the method of Maio and Rickenberg(5) and bioassay with lysineless mutant #26-26 of *E. coli.* Inocula for the bioassay were grown for 18 hours in Davis-Mingioli medium with 2×10^{-4} M lysine(6) accompanied by a control tube without lysine. Washed *E. coli,* approximately 5×10^6, were added to 2-fold serial dilutions of the Krebs 2 supernatants which had been freed of coagulable protein. Standards containing known concentrations of lysine in D-M medium were set up for each determination. After incubation, with periodic shaking for 5 to 7 hours at 36°C the turbidity of the unknowns and standard was compared in a Klett colorimeter. Extracts obtained from cells which had been incubated 4 to 18 hours without lysine gave levels of lysine slightly higher than those obtained with trichloracetic acid but at 0.3-0.5 mM were comparable to free lysine measurements done by other methods with various mammalian cells(7,8). Egress as well as entry of lysine could be demonstrated by incubating the cells first with high concentrations then washing and continuing incubation in a medium without lysine.

Results. Kinetics of lysine transport and inhibition of protein synthesis. When Krebs 2 cells are incubated in the simplified medium protein synthesis is linear for the first few hours but the rate decreases gradually between 6 and 18 hours probably because of depletion of the intracellular amino acid pool. Addition of a large excess of lysine to the medium stops protein synthesis at 2 to 6 hours depending on the concentration (Fig. 1). At lower levels of lysine the cells show some ability to concentrate this amino acid in the interior giving a distribution ratio (intracellular/extracellular) of 2 or greater (7). With higher concentrations the distribution ratio approaches unity but there is no ability to exclude lysine and the intracellular concentration may reach 100 times normal. With external concentrations of 15 mM or less lysine continues to enter the cells for as long as 18 hours. Although incubation with the highest experimental concentration gives a very high intracellular level at 4 hours, less is found at 18 hours probably due to cell damage and loss of lysine during the washing of cells. Protein synthesis stops when the intracellular lysine level approaches 20 mM and external concentrations below 7 mM (about 1.0 mg/ml) being unable to produce this internal concentration have little or no effect on protein synthesis. Addition of lysine 4 hours after the leucine C^{14} results in a degree of inhibition of total protein synthesis about half that obtained with zero-hour addition.

Reversal of lysine inhibition. As previously reported a number of amino acids will reverse

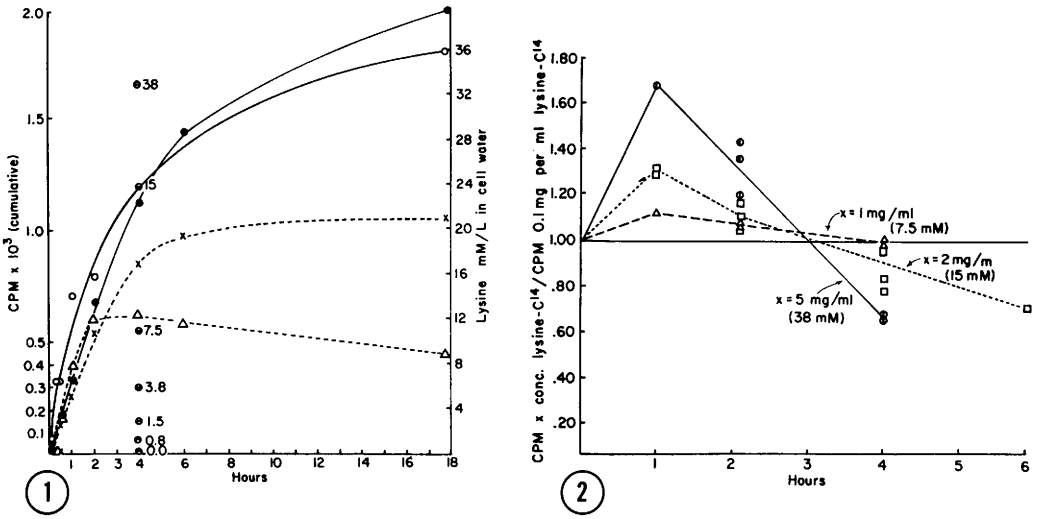

FIG. 1. Relation between free intracellular lysine (right-hand scale) and protein synthesis as measured by uptake of leucine C^{14} (left scale). CPM is activity of sample representing 2.5 mg wet weight of cells. ○—— intracellular lysine resulting from 15 millimolar external concentration; ⊖ intracellular concentration at 4 hr with extracellular concentration in mM as indicated by corresponding numbers. Leucine C^{14} uptake: ●—— controls; △----- 38 mM lysine, ×----- 15 mM lysine.

FIG. 2. Effect of external concentration on uptake of lysine C^{14} into TCA precipitate. Lysine C^{14} with specific activity of 10^6 CPM/mg added at the concentration indicated by X: 5 mg with 5×10^6 CPM per ml, 2 mg with 2×10^6 CPM per ml. Results are given as ratio to lysine C^{14} uptake in "normal" protein synthesis as measured with 0.1 mg and 10^4 CPM per ml (1.00 on the graph).

lysine inhibition of viral replication(1,2). It was found that these same amino acids also reverse the inhibition of cell protein synthesis. Inhibition produced by lysine at concentrations of 5.0 mg/ml (38 mM) was significantly reversed by alanine, valine, methionine, proline, hydroxyproline, glycine, all at 1 mg/ml, as well as by amino acids not found as constituents of protein; dl-norvaline, and α-amino-n-butyric acid. With lysine at 2 mg/ml (15 mM) these and also isoleucine, phenylalanine and dl-homoserine some at concentrations as low as 0.2 or 0.05 mg/ml reversed the inhibition of cell protein synthesis. The d-isomers were much less effective as reversers and some of the corresponding α-keto acids were tried without effect.

Representative results of these experiments are presented in Table I. Quantitatively the inhibition of cell protein synthesis was always relatively small compared with the reduction of viral HA titers by lysine at 2.0 mg/ml. Isoleucine at 0.2 mg/ml, proline at 0.05 mg/ml and certain other amino acids almost completely reversed the inhibition of protein synthesis while the HA titers were still a small fraction of controls. Amino acids such as proline which were by themselves slightly inhibitory produced more significant reversal at lower concentrations. α-Amino isobutyric acid was unique in that it failed to act as a reverser on cell protein synthesis which it inhibited at 1.0 mg/ml, but did produce slight and somewhat irregular reversal of viral inhibition by lysine.

Synergistic inhibition by histidine with lysine. At concentrations of 0.5 mg/ml, l-histidine was not inhibitory to protein synthesis or HA formation as may be seen in the last column of Table II. However, when histidine was combined with lysine at 1 or 2 mg/ml uptake of leucine C^{14} or phenylalanine C^{14} was well below that with lysine alone and some inhibitory effect on protein synthesis was found with the combination at concentrations as low as histidine 0.1 mg/ml plus lysine 0.5 mg/ml. These are well below the minimal inhibitory levels of these amino acids individually. In the presence of lysine 1.0 mg/ml HA inhibition was observed with his-

TABLE I. Reversal of Lysine Inhibition of Protein Synthesis and Viral Replication.

Reversing amino acid	mg/ml	CPM,* lysine 2.0 mg/ml	R‡	HA cells,† lysine 2.0 mg/ml	R‡
l-isoleucine	1.0	p-600	.95	720	2.8
	.2	p-610	.97	128	.17
	.05	p-335	.65	64	.25
	0	p-246	.38	4	.016
l-proline	1.0	p-380	1.25	64	.66
	.2	p-370	.90	128	1.0
	.1	—	—	384	3.0
	.05	p-510	.98	64	.16
	0	p-330	.63	6	.016
dl-norvaline	1.0	l-1080	1.06	225	1.2
	.2	l-660	.56	40	.04
	0	l-470	.41	16	.01
α-amino-isobutyric acid	1.0	l-570	.90	52§	.81
	.2	l-405	.40	16	.12
	0	l-515	.50	8	.01

* Counts per min on trichloracetic acid precipitate after 18 hr. Sample represents 2.5 mg wet wt of cells. p = phenylalanine C^{14}, l = leucine C^{14}.

† Reciprocal of hemagglutinin titers of cell extracts at 48 hr. (Geometric mean from several experiments.)

‡ R = ratio to control with reversing amino acid but without lysine.

§ α-Amino-isobutyric acid at 0.5 mg/ml.

tidine down to 0.1 mg/ml but there was little effect on HA at the next lower concentration of lysine. With 2 other basic amino acids, arginine and ornithine, in combination with either histidine or lysine there was much less evidence of synergism.

Effect of reversing amino acids on lysine transport. Several of the reversing amino acids reduced the amount of lysine found by bioassay of the intracellular pool of free amino acids. This reduction was of similar percentage at 4 and 18 hours. The results presented in Table III are a summary in which the effects on protein synthesis, HA formation and lysine transport are represented as the ratio of the value for lysine plus the reversing amino acids to that of lysine alone. The reversing amino acids are arranged approximately in order of their ability at 1.0 mg/ml to decrease entry of lysine into Krebs 2 cells. Of the several tested only histidine produced a definite increase in the uptake of lysine into the free amino acid pool. Four other amino acids, ornithine, arginine, aspartic acid and threonine (not included in Table III) which did not reverse lysine inhibition had no effect on transport of this amino acid.

In general there appeared to be some correlation between effectiveness as reversers and ability to inhibit transport of lysine but with the lower concentrations of the amino acids listed in Table III several exceptions may be noted. Thus, alanine at 0.2 mg/ml had no effect on lysine transport (ratio 1.0) but it increased the HA titer 5-fold over that with

TABLE II. Synergistic Inhibitory Effects of Lysine and Histidine.

Histidine, mg/ml	Lysine, mg/ml 2.0	1.0	0.5	Ornithine, mg/ml 1.0	Control
A. Cell-protein synthesis: CPM at 18 hr					
1.0	l-225	—	—	800	940
.5	l-265	420	560	880	1130
.2	l-400	570	605	1050	1140
.1	l-810	800	1070	—	—
0	l-485	1200	1265	1340	1280
.5	p-145	—	—	1275	1620
.2	p-400	800	1100	1275	1830
0	p-517	1420	1650	1800	1700
B. Hemagglutinin titers at 48 hr					
Histidine					
1.0	<4	8	128	32	1024
.5	<4	10	256	256	2048
.2	<4	20	256	256	1024
.1	<4	8	512	—	1024
Arginine					
1.0	8	128	256	32	512
Ornithine					
1.0	4	128	64	—	256
0	<4	512	512	256	1024

TABLE III. Effect of Amino Acids on Lysine Transport as Related to Reversal of Lysine Inhibition.

Protein synthesis 18 hr*

Amino acid	Conc, mg/ml	Leucine-C^{14}	Phenylalanine-C^{14}	Hemagglutinin formation (48 hr)	Inhibition of lysine transport* at 4 hr
α-amino-isobutyric acid	1.0	1.0	1.1	4	1.0
l-alanine	1.0	3.0	2.1	16	.70
	.2	2.4	1.0	5	1.0
β-alanine	1.0	1.4	1.1	6	1.0
l-valine	1.0	2.3	2.0	32	.33
	.2	3.5	1.2	3	1.0
d-valine	1.0	1.6	1.3	4	.75
dl-serine	1.0	1.2	1.0	3	.50
dl-norvaline	1.0	2.3	1.9	14	.50
	.2	1.4	1.2	2.5	.80
l-isoleucine	1.0	2.4	2.4	180	.40
	.2	2.0	2.4	32	.60
	.05	1.1	1.4	16	1.0
l-proline	1.0	1.2	1.1	11	.33
	.2	1.7	1.1	21	.35
	.05	2.1	1.5	11	.87
l-histidine	1.0	.47	.20	(.016)†	>2 (2.0)
	.2	.82	.78	(.04)	>2 (1.4)
	.1	1.6	—	(.016)	2.0(1.2)

* All results are expressed as the ratio: value (CPM, HA or mM lysine) with reversing amino acid + lysine/value with lysine alone. When there is no reversal the result is 1.0; reversal >1.0; inhibition <1.0.

† In parentheses, result with 1.0 mg/ml lysine.

lysine alone. Alanine also reversed lysine inhibition of the uptake of leucine C^{14} into the acid insoluble fraction although this was not the case with uptake of phenylalanine C^{14}. A similar result was obtained with valine 0.2 mg/ml. At concentrations of 1.0 mg/ml, dl-serine and l-isoleucine reduced lysine transport by about 50% yet isoleucine was superior to serine as a reverser, both of protein synthesis and HA formation. Proline was the strongest antagonist of lysine transport but less effective than isoleucine as a reverser.

Incorporation of lysine C^{14} into the protein-nucleic acid fraction. The possibility was considered that excess lysine might be combined in certain cell or virus-related proteins resulting in abnormal enzyme structure and interference with cell metabolism and virus synthesis. If this were so the excess incorporation of lysine might be detected as more C^{14}-labeling with increasing concentrations of lysine of constant specific activity. Lysine at a specific activity of 10^5 CPM/mg was added in a range of concentrations from 0.1 mg/ml to 5.0 mg/ml and counts were done on the cold TCA precipitate at 1, 2, and 4 hours. No significant differences were found with external lysine concentrations of 0.1 to 0.5 mg/ml. Because of the considerable dilution of isotope, counts obtained at 4 hours in several experiments averaged only 207 ± 35 CPM for a 5 mg (wet weight) sample of cells. A few measurements of free lysine C^{14} in the acid soluble fraction gave values comparable to those obtained by bioassay and several times the CPM of the protein fraction. With the highest concentrations of lysine, 2 or 5 mg/ml, excess radioactivity in the TCA precipitates was measurable at 1 and 2 hours. The maximum appeared at 1 hour where 64 CPM were found with 5 mg/ml lysine, 49 CPM with 2 mg/ml, as compared with 38 CPM in controls containing 0.1 mg/ml of lysine. The resulting ratios of excess labeling were 1.68 and 1.30, respectively. At 2 hours with 5 mg/ml of lysine, ratios significantly higher than 1.0 also were seen (Fig. 2) and there was some evidence of excess labeling at lower external concentrations. At 4 to 6 hours when pro-

tein synthesis was diminished or stopped by inhibitory concentrations of lysine less label was incorporated than at 0.1 mg/ml and the resulting ratios became less than 1.0. Although the absolute values of C^{14} uptake varied from one experiment to another the ratios between values for excess lysine and controls in each experiment were reproducible.

Discussion. Uptake of lysine into the free amino acid pool of Krebs 2 ascites tumor cells is slower but more persistent than has been reported with other amino acids(9) which reach equilibrium in a few minutes, and uptake of lysine at 5-20 mM into cells of the rat intestine *in vitro* shows less reduction by isoleucine than in K2 cells. Also of interest(9) is the inhibition of amino acid transport by related compounds that are not constituents of proteins, and increase of lysine uptake in the presence of glutamate. The latter was used as the sole source of nitrogenous nutrient in our minimal medium.

With the several amino acids investigated in Krebs 2 cells there was a reasonable correlation between ability to reduce intracellular concentrations of lysine below a critical level and reversal of inhibition, but on a quantitative basis several exceptions have been noted. At the lower concentrations of some of the amino acids there was little effect on lysine transport, but protein synthesis returned close to normal and partial reversal of viral inhibition was also obvious. A recent report on reversal of guanidine inhibition by amino acids(10) is of interest and transport effects should be investigated. It is well known that with certain combinations of 2 amino acids transport of one or both may be increased and the synergistic effects of histidine on lysine inhibition may be explained on this basis.

Since the mode of action of lysine is unknown additional action of reversers at a site other than the cell membrane cannot be excluded. Interpretation of the uptake into the cold TCA precipitate of excess C^{14} from lysine added at inhibitory concentrations must await further investigation to determine whether the label is in lysine or a metabolic product and in what fraction it is to be found. The excess uptake preceded the inhibition of protein synthesis and since rate of labeling later decreased, at a time when the concentration of free intracellular lysine was increasing (Fig. 1 and 2), the finding of excess lysine in the TCA precipitate cannot be attributed to failure of removal of free lysine by washing. Irrespective of whether the label is due to lysine or a metabolic product the results are of interest in suggesting abnormal protein or nucleic acid synthesis.

Evidence has been presented in a previous paper(2) that lysine and some other inhibitory amino acids when added at concentrations below the toxic level act only on an early stage of virus replication. The lysine-sensitive event may be the synthesis of a viral precursor, protein or enzyme, which is completed within the first 3 hours. Addition of lysine at 15 mM several hours after the virus has little effect on replication of hemagglutinins although cell protein synthesis is still inhibited to some extent. From the kinetics of inhibition and reversal there is evidence that the preliminary synthesis of virus related proteins during eclipse is far more vulnerable to amino acid excess than cell protein synthesis or even the later phase of HA replication.

Summary. Reversal or augmentation by certain amino acids of lysine inhibition of viral replication and cell protein synthesis is related to effects on transport of lysine. Interaction of excess lysine or a metabolic product with the protein-nucleic acid fraction of cells is indicated by observations on C^{14} labeling. These results plus previous evidence suggest that formation during the eclipse period of a viral precursor is significantly more sensitive to excess lysine than synthesis of cell protein or influenza virus structural protein as represented by hemagglutinin.

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Effect of 1,3-*Bis*-(2-Chloroethyl)-1-Nitrosourea on *Saccharomyces cerevisiae*.* (30361)

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Schabel *et al*(1) reported the effects on L1210 leukemia of a number of substituted nitrosoureas synthesized by Johnston *et al* (2). Of those tested, 1-(2-chloroethyl)-1-nitrosourea (NSC-47547) and 1,3-*bis*-(2-chloroethyl)-1-nitrosourea (NSC-409962; BCNU) were markedly effective when the tumor was inoculated by the intraperitoneal or intracerebral route. Cross-resistance studies using plasmacytoma in hamsters suggested a biochemical mode of action similar to that of alkylating agents. The authors pointed out, however, that the 2-chloroethyl groups on these compounds would not be expected to form ethylenimonium ions *in vivo*, and are not essential for activity of the parent 1-methyl-1-nitrosourea. In clinical trials of BCNU, Rall *et al*(3) found that even though toxicity was rather severe, the drug decreased the number of circulating leukemic cells and was uniquely effective in clearing meningeal leukemia when given orally. More recently Goldin *et al*(4) confirmed the effectiveness of BCNU against a number of experimental leukemias, emphasizing its extensive activity over a wide range of treatment schedules, and suggested that studies of the mechanism of action of this drug be undertaken.

The data obtained previously on hydroxyurea in a microbial test system(5) were compatible with other studies on mechanism of action of that drug(6-9). Consequently a similar study was initiated with BCNU; a report follows.

Materials and methods. The 1,3-*bis*-(2-chloroethyl)-1-nitrosourea (NSC-409962; BCNU) and 1-methyl-1-nitrosourea (NSC-23909; MNU) were supplied by Dr. Harry B. Wood, Jr. and Dr. J. A. R. Mead of the Cancer Chemotherapy National Service Center, and were stored at —20°C. Mechlorethamine hydrochloride (nitrogen mustard, HN2) was obtained from Merck and Co., Inc. All microorganisms used were stock laboratory strains maintained in this laboratory.

Initial tests of inhibition of microorganisms were done on agar plates on which a filter paper disc impregnated with a 1% solution of each compound was placed. Measurements of dose-response relationships were done in Sabouraud dextrose broth (Difco) dispensed in matched culture tubes. Growth was expressed as optical density at 640 λ after establishing linearity of optical density with cell population. Nephelo-culture flasks with 19 mm diameter side arms (Bellco Glass Co.) were used in measurements of antagonism of inhibition; growth was expressed as optical density at 640 λ as above. Nucleic acid and protein determinations were performed with minor modifications of the procedures described earlier(5); such modifications were used only to compensate for the relatively low concentration of deoxyribonucleic acid (DNA) in yeast. Measurements of carbon to chlorine bonds were done by a

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