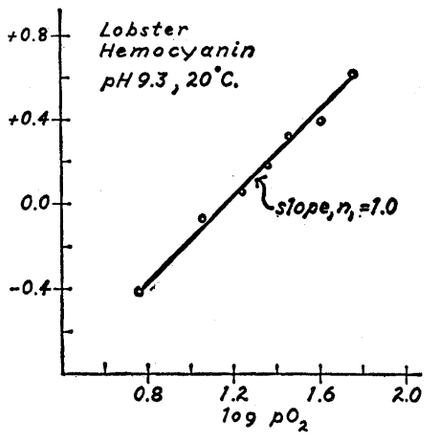

Fig. 3. Oxygen equilibrium data obtained in a Ca^{2+}-free glycine buffer of *p*H 9.3 and ionic strength 0.07, at 20°C. Ordinate is $\log[y/(1-y)]$; y is the fractional degree of oxygenation, and pO_2 is the oxygen pressure.

molecular weight unit seen by Levin (*1*) in electron micrographs of material prepared at *p*H 10.2. These observations indicate that the smallest functional molecule is composed of two subunits.

In amino acid analysis of lobster hemocyanin by standard procedures (*10*) (Table 1), approximately 15 residues each of lysine and arginine were found in a unit having a molecular weight of 34,000. Almost identical analyses were reported for the hemocyanin of the very similar species, *Homarus vulgaris* (*11*). Tryptic peptides from lobster hemocyanin have been separated by automatic column chromatography procedures (*12*). A column (0.9 by 15 cm) of Spinco type 50A resin was used at 50°C with a 250-ml linear gradient composed of pyridine and acetic acid starting at *p*H 3.1, 0.2*M* pyridine; and ending at *p*H 5.0, 2.0*M* pyridine. Thirty-one tryptic peptides have been isolated, of which 14 are arginyl and 17 are lysyl. Most of the peptides contain histidine. It is significant that a "fingerprint" pattern of a tryptic peptide from another crustacean, *Palinurus vulgaris*, also shows about the same number of peptides (*11*). Although the determination of the amino acid composition of each tryptic peptide is not yet complete, this result is consistent with the hypothesis that the minimum functional unit (68,700) consists of two subunits which are very similar and perhaps identical. Since every peptide contains either arginine or lysine, nonspecific cleavage by trypsin does not appear to be a problem.

A molecular weight of 825,000 was previously found for lobster hemocyanin when sedimentation and diffusion coefficients and partial specific volume measurements were used (*13*). We assume that the molecule is composed of 12 subunits, each of molecular weight 68,700. However, the copper content (*14*) of 21 of our samples was 0.175 ± 0.008 percent by weight. The minimum functional unit on this basis would have a molecular weight of 72,600. No significant loss of copper was observed after prolonged dialysis except in calcium-free buffers at *p*H 9.3. Under these conditions hemocyanin slowly lost about one-half of its copper. This observation suggests that the two copper atoms in each binding site may be linked to the protein differently.

If the 4*S* unit contains two polypeptide chains and two copper atoms, one copper atom might be bound by each chain. Each pair of copper atoms is associated with the binding of one oxygen molecule. Perhaps, therefore, each oxygen molecule is bound at a site between two polypeptide chains.

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Actinomycin D: Inhibition of Respiration and Glycolysis

Abstract. *Actinomycin D inhibited respiration and anaerobic glycolysis of human leukemic leukocytes and lowered the adenosine triphosphate content of the cells. Inhibitory effects on respiration and on RNA synthesis could not be dissociated from one another over a wide range of drug concentrations. Actinomycin D also impaired protein synthesis, probably by decreasing the availability of adenosine triphosphate and by inhibiting messenger RNA.*

Actinomycin D, a chromopeptide produced by actinomycetes, has been shown to selectively inhibit the DNA-directed synthesis of RNA (*1*); it selectively binds to a native DNA template (*2*). Several recent observations, however, have suggested that actinomycin D may also produce subtle cellular changes that are not explained by inhibition of RNA. Revel, Hiatt, and Revel (*3*) reported progressive inhibitory effects on the synthesis of protein in rat liver after RNA synthesis had been blocked, and Korn (*4*) found that the drug impaired phage maturation without detectable effects on RNA. During our studies on the metabolism of human leukemic cells, we observed significant inhibition of glycolysis and respiration in the presence of actinomycin D.

Human leukemic leukocytes were prepared for testing by the following procedure: 50 ml of heparinized blood was allowed to settle at 4°C, the leukocyte–plasma phase was removed, and crystalline glucose (2 mg/ml) and sodium bicarbonate (1 mg/ml) were added. For studies of energy metabolism (inhibition of oxygen consumption and carbon dioxide production) cell suspensions (2.5 ml) were pipetted into manometer flasks and incubated with shaking at 37°C. Actinomycin D was dissolved in Krebs-Ringer bicarbonate medium and tipped into the

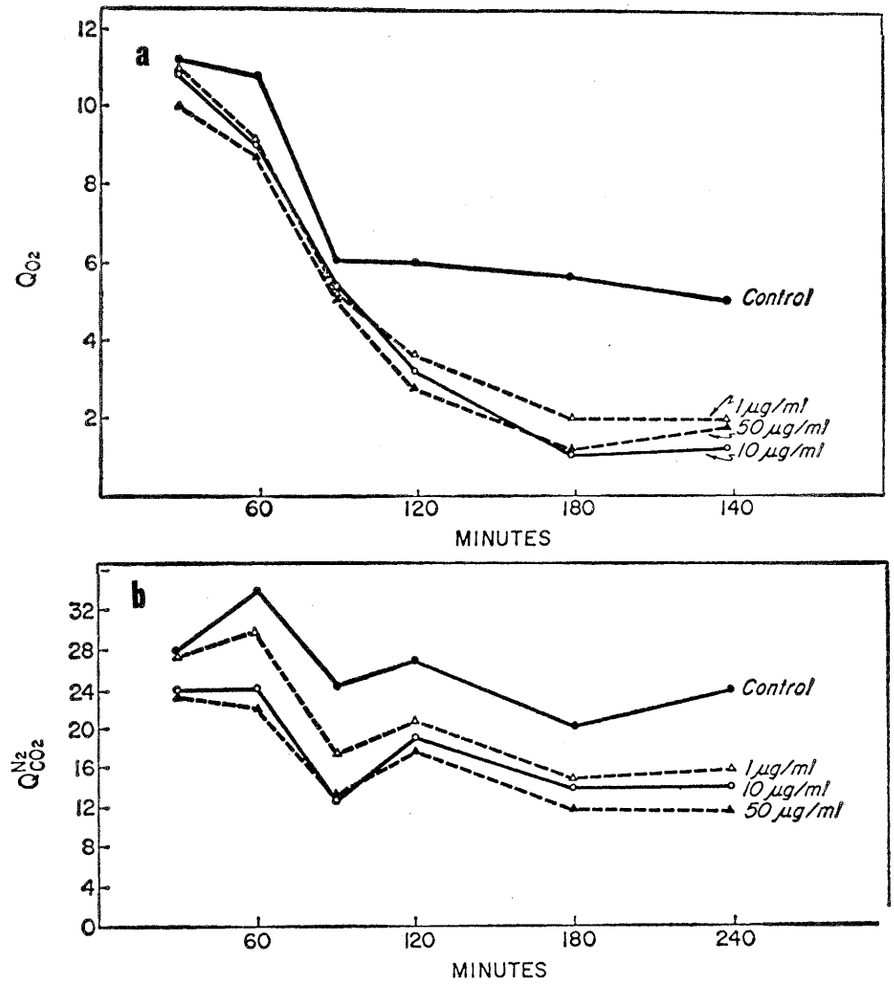

Fig. 1. Respiration (*a*) and anaerobic glycolysis (*b*) of leukemic lymphocytes. Actinomycin D, in concentrations of 1, 10, or 50 μg/ml, was tipped into the main compartment of each vessel, which contained 2.5 ml of cell suspension (8.0 × 10^7 cells/ml), after equilibrium on the bath. Data are expressed as oxygen consumption and carbon dioxide production per milligram dry weight of cells per hour.

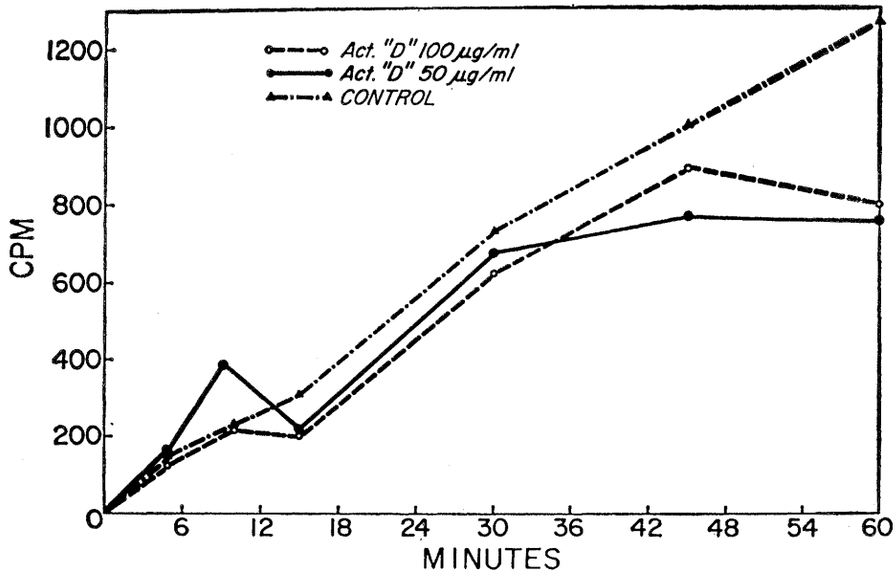

Fig. 2. Protein synthesis of leukemic lymphocytes measured as incorporation of arginine-^{14}C into polypeptide precipitable by trichloroacetic acid. Each flask contained 1 × 10_8 cells/ml; actinomycin D was added in concentrations of either 50 or 100 μg/ml.

Table 1. Effect of actinomycin D on the concentration of adenosine triphosphate (ATP) in human leukemic lymphocytes. Six tubes of cell suspensions containing 6.0 × 10^7 cells/ml were incubated in a mixture of oxygen (95 percent) and carbon dioxide (5 percent) for 3 hours at 37°C. Actinomycin D was added at time zero to tubes 4, 5, and 6. At the end of the incubation period, the contents of each tube were assayed for ATP.

Tube No.	ATP (mμmole/ml)
Control cells	
1	39.0
2	29.5
3	36.0
	Av. 34.8
Cells plus actinomycin D (50 μg/ml)	
4	3.8
5	2.2
6	4.9
	Av. 3.6

main compartment from the vessel sidearm at zero time. Determinations of respiration, aerobic glycolysis, and anaerobic glycolysis were performed with standard Warburg manometry. Adenosine triphosphate (ATP) was determined by means of the luciferase assay (*5*).

Incorporation of amino acids into polypeptides was measured by incubating 4 ml of cell suspension in the presence of 5 μc of arginine-^{14}C at 37°C for 60 minutes. Duplicate samples (0.1 ml) were removed at 10-minute intervals, rapidly dried on Whatman filter discs, and washed twice in cold 5 percent trichloroacetic acid. The discs were heated in 5 percent trichloroacetic acid at 90°C for 7 minutes; washed first in ethanol, then in a mixture of ethanol, chloroform, and ether (2 : 2 : 1); and finally in ether. The discs were dried and counted in a scintillation counter.

Incorporation of precursor into RNA was measured by incubating 2.5 ml of cell suspension at 37°C for 2 hours in the presence of 15 μc of orotate-^{14}C. Duplicate samples (0.1 ml) were removed at intervals during the incubation and rapidly dried on Whatman filter discs which were then immersed in cold 10 percent trichloroacetic acid. The discs were washed in Hokin's solution (*6*) at 37°C for 30 minutes, then in a mixture of Hokin's solution and ether (1 : 1) at 37°C for 30 minutes, and finally rinsed in ether.

Our experiments showed that actinomycin D inhibited both respiration and glycolysis of leukemic cells. The minimum quantity of the drug necessary to produce inhibition depended upon the concentration of cells in the re-

Table 2. Effect of actinomycin D and puromycin on respiration and protein synthesis. Oxygen uptake is expressed as total microliters consumed per vessel during a 5-hour incubation (2.5 ml of cell suspension per flask containing 6.0×10^7 cells/ml). Polypeptide synthesis was determined on cell samples containing 1.25 μc arginine-^{14}C/ml. Both actinomycin D and puromycin were added at time zero.

Sample	Oxygen uptake (μl)	Polypeptide synthesis (count/min)
Control cells	156	2255
Cells plus actinomycin D (50 μg/ml)	118	1224
Cells plus puromycin ($1 \times 10^{-4}M$)	153	175

action flask (representative leukocyte studies are shown in Fig. 1). Leukemic lymphocytes sampled at the end of 3 hours of incubation with actinomycin D had significantly decreased levels of ATP (Table 1). The incorporation of amino acids into polypeptides was inhibited within 45 minutes by actinomycin D, whereas incorporation in the controls remained constant for 1 hour (Fig. 2).

In view of the inhibitory effects of actinomycin D on respiration, anaerobic glycolysis, and polypeptide synthesis, experiments were designed to determine whether these effects could be dissociated from the action of the drug on RNA synthesis. Respiration and RNA synthesis were either inhibited together or not inhibited at all over a wide range of drug concentrations (Fig. 3). The effect of puromycin was then tested in order to determine if the effect of actinomycin D on respiration was mediated primarily by an action on RNA synthesis leading to impaired synthesis of respiratory enzymes. However, concentrations of puromycin sufficient to completely block protein synthesis ($10^{-4}M$) failed to influence leukemic cell respiration (Table 2).

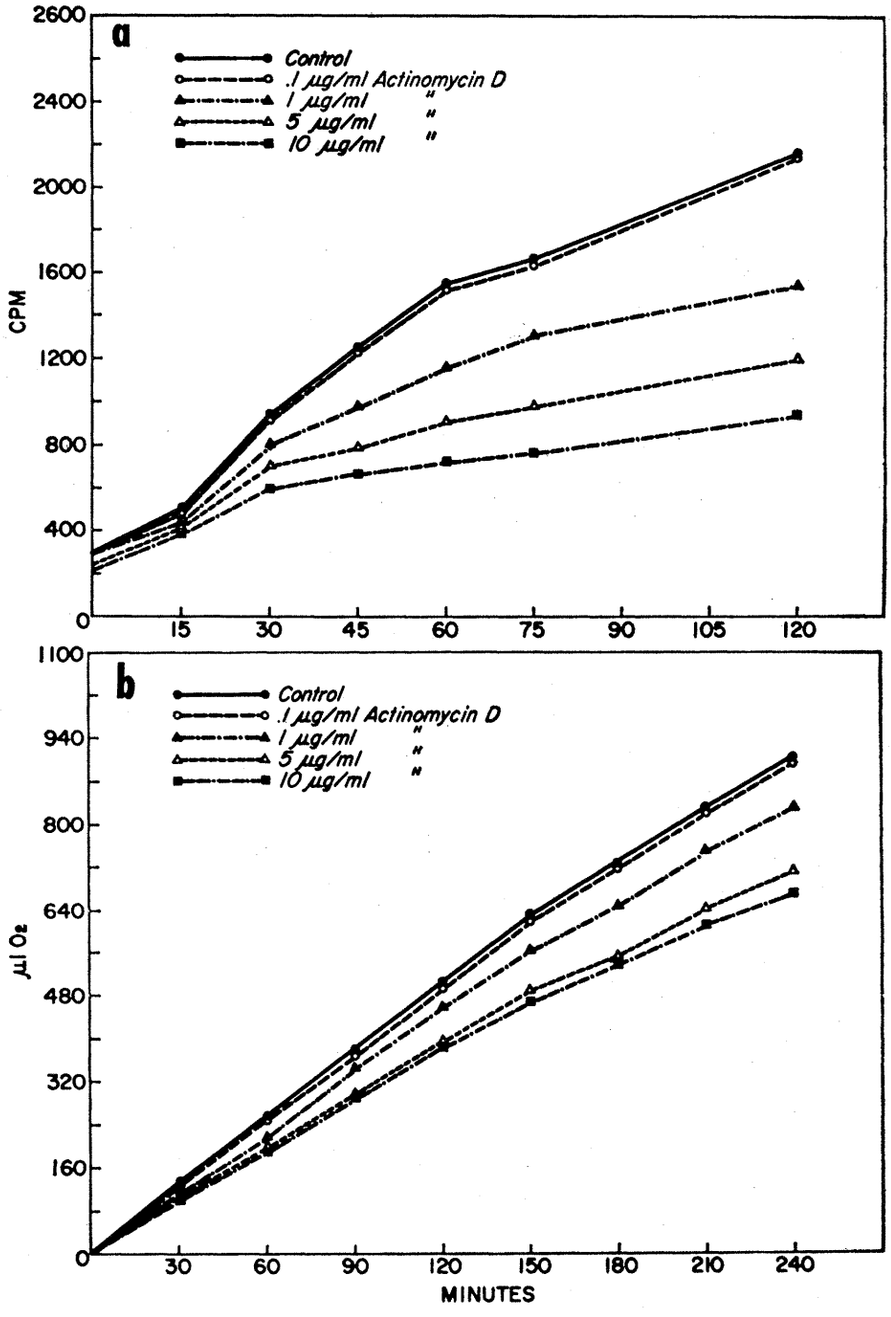

Fig. 3. Comparative inhibition by actinomycin D of RNA synthesis (*a*) and respiration (*b*) in leukemic myeloblasts (3×10^6 cells/ml). Actinomycin D was added to the cell suspensions in concentrations of 0.1, 1, 5, or 10 μg/ml.

Thus, respiration and glycolysis of human leukemic leukocytes (both lymphocytes and granulocytes) were depressed by the presence of actinomycin D. This inhibition was reflected by decreases in the concentrations of cellular ATP to 10 percent of untreated controls. Furthermore, we were unable to find concentrations of actinomycin D which inhibited RNA synthesis without affecting respiration and glycolysis, although the effects on RNA were detected earlier and were slightly greater in magnitude. Direct inhibition of protein synthesis should block energy metabolism if the effect of actinomycin D were due to impaired RNA synthesis coding for respiratory and glycolytic enzymes. Our data showed that, unlike actinomycin D, puromycin failed to alter respiration despite complete inhibition of protein synthesis.

Although the exact mechanism has not been resolved, it is clear that actinomycin D inhibits respiration and glycolysis of human leukemic cells. These preliminary experiments also suggest that inhibition of protein synthesis in intact cells by actinomycin D may be mediated by at least two separate mechanisms: (i) specific binding of actinomycin D to DNA with resultant inhibition of messenger RNA transcription, and (ii) impairment of energy metabolism causing a decrease in ATP available for the formation of active amino acyl transfer RNA.

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6. Hokin's solution: 935 ml ethanol, 60 ml glacial acetic acid, and 4 ml of 2*N* NaOH.

7. Supported by the American Cancer Society (P-363) and NSF (GB-1416). D.S.M. was a PHS trainee (T4-CA-5042). We thank Clarence Ellis for technical assistance, George Boxer for providing the actinomycin D, and T. J. McManus for performing the ATP assay.

8. Since this paper was submitted it has been reported that the addition of glucose reversed actinomycin D–induced inhibition of protein synthesis in ascites cells [G. R. Honig and M. Rabinovitz, *Science* **149**, 1504 (1965)]. The authors concluded that this effect was independent of RNA synthesis. Lack of data on energy metabolism of these ascites cells precludes a direct comparison with data in this report.

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Transport of Sugars and Amino Acids in the Intestine: Evidence for a Common Carrier

Abstract. D-*Galactose,* L-*arginine, and their respective actively transported analogs are partially competitive inhibitors of the active transport of neutral amino acids in the small intestine of hamsters. Since the aforesaid classes of compounds are all transported by similar, sodium-ion-dependent mechanisms and elicit countertransport of each other, all may share a common, polyfunctional carrier in which a series of separate binding sites, namely, one each for sugars, neutral amino acids, basic amino acids, and* Na^+ *are joined together, as in a mosaic.*

The small intestine can actively transport substances as different as sugars, amino acids, pyrimidines, and bile salts by similar, Na^+-dependent, stereospecific processes. In all cases, the net movement, including direction and extent, of the substrates into or out of the tissues is determined by the Na^+ gradient between the medium and the cells; that is, the process is freely reversible, and its net direction may be reversed by inverting the Na^+ gradient. Transport may require formation of ternary complexes of substrate, Na^+, and a membrane "carrier" (*1*). The carrier has not been identified, but it presumably is, or involves, protein (or proteins) with specific binding sites, and is "mobile"; that is, it is capable of providing access of bound, water-soluble substrates alternately to the extra- and intracellular fluids, across the lipidic barrier of the membrane (*2*). In principle, there should be at least as many different binding sites as there are classes of transported compounds and, consequently, as tacitly assumed by most workers, as many "carriers." However, recent work suggests an alternative hypothesis.

In 1926 Cori (*3*) suggested that sugars and amino acids may be absorbed "at the same place of the cell structure." Recently, reciprocal interactions between various Na^+-dependent transport mechanisms in the intestine have been noted. For instance, some sugars inhibit amino acid transport (*4, 5*), and some amino acids inhibit sugar transport (*6*). Also, although individual carrier systems have been postulated for the transport of different groups of amino acids, such as neutral, basic, acidic, and *N*-substituted (*7*), significant cross-inhibition between the different groups occurs (*8*). Some of the characteristics of the inhibition of transport of neutral amino acids by sugars and by basic amino acids are reported here.

Rings of everted hamster small intestine were incubated (*9*) with either C^{14}-cycloleucine (1-aminocyclopentane-1-carboxylic acid), which was assayed in trichloroacetic acid extracts of tissues and media with a liquid scintillation spectrometer, or with L-tyrosine, which was determined as phenol (*10*). Complete experimental procedures, including methods for demonstrating countertransport and for processing and presenting the data, have been described (*11*).

When the concentration of inhibitor (I) is constant and that of substrate (S) is varied, both D-galactose (Fig. 1) and L-arginine (not shown) competitively inhibit cycloleucine transport. In order to distinguish between fully competitive and partially competitive inhibition (*12*), the substrate concentration was kept constant and the inhibitor concentration was varied (Fig. 2). According to this method, both galactose and arginine behave as partially competitive inhibitors of cycloleucine transport, in contrast to the neutral amino acids—L-histidine, L-proline, and L-methionine—which behave as fully competitive inhibitors (*6*). From experiments of the first type (Fig. 1), kinetic constants for cycloleucine transport have been calculated. The average of six experiments gave the following constants, corrected for the extracellular space (*13*): K_m (the Michaelis constant, presumably identical with the dissociation constant of the substrate-carrier complex) was 5.15 mmole/liter (range: 3.8 to 6.0); and V_{max} (the maximum transport rate in conditions of saturation with substrate and with Na^+) was 2.74 μmole per milliliter of tissue water per minute (range: 2.2 to 2.9). These values and an average substrate concentration of 2.54 m*M* were used to calculate the theoretical curves shown in Fig. 2. Both curves *a* (Fig. 2) were calculated on the assumption of fully competitive inhibition (*12*, Eq. VIII.7) and a K_i (inhibition constant or dissociation constant of the inhibitor-carrier complex) of 14.5 mmole/liter. Curve *b* was calculated for partially competitive inhibition (*12*, Eq. VIII.28) and a K_i of 2.5 mmole/liter. Curve *b* fits the experimental data. Furthermore, when the ratio V_{max}/v (maximum rate/initial rate) is plotted against the inhibitor concentration (inset, Fig. 2), a parabolic curve rather than a straight line is obtained, as expected from the equations of partially competitive inhibition (*12, 14, 15*). A similar result was obtained with arginine as the inhibitor, whereas the neutral amino acids, L-histidine, L-proline, and L-methionine, acted as fully competitive inhibitors and gave straight lines.

If current enzyme kinetic theory is applied to the membrane carrier problem, a partially competitive inhibitor may be construed as a substance that binds to a site different from, but close to, the catalytic or substrate-binding site, so that a change in the apparent affinity of the catalytic site for its substrates, without a change in V_{max}, is induced. Such effects may properly be classified as "allosteric" (*12, 15, 16*) and operationally appear as a change in K_m to a higher, limiting value, K_m'. Partially competitive inhibition may be measured by the ratio K_m/K_m', which by definition is smaller than one (*17*).

From these considerations, two conclusions seem warranted. First, all the neutral amino acids studied (cycloleu-