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ABSTRACT

Leukocytes, isolated from peripheral blood and shown to be completely viable, were used in the detection of metabolic changes in the following pathological cases:

a) In chronic myelogenous leukemia, to measure the alteration of the metabolism of the uniformly ^{14}C-labelled L-asparagine by the leukemic cells.

b) In schizophrenia, to detect the changes in the oxidation of ^{14}C-labelled methyl carbon atom of L-methionine and to study the effect of chlorpromazine on the metabolism of this amino acid.

Leukocytes from chronic myelogenous leukemic (CML) patients were incubated in a medium containing uniformly ^{14}C-labelled L-asparagine, and the label was traced in some of the metabolic products. In cells from the CML patients the uptake and incorporation into proteins of this amino acid were found to be significantly higher than in cells from the individuals with no leukemic syndrome. These differences were shown not to be due to pool size effects. Also, the independence of chronic myelogenous leukemic leukocytes on L-asparagine was demonstrated.

Differences were established in the oxidation of the methyl carbon atom, but not of the carboxyl one, of L-methionine by the leukocytes of psychiatric patients and control subjects. The patients could not be separated from the controls according to the incorporation of the label from either L-methionine-methyl-^{14}C or L-methionine-carboxyl-^{14}C into cellular proteins. These findings support the hypothesis claiming involvement of disturbances of methylation processes in schizophrenia.

Chlorpromazine was shown to have varying dose-response effects on the viability of leukocytes. It was found to inhibit in vitro protein synthesis in the cells of some subjects but to have no effect on the cells of others, indicating differing sensitivity of the leukocytes of various individuals to this tranquillizer.

I. INTRODUCTION

With the advent of improved techniques in their separation from human blood, leukocytes have gained popularity in clinical investigations in vitro. Their easy availability from peripheral blood and their possession of the major cell organelles make them elegantly suitable as a biopsy specimen.

Isolated leukocytes can be incubated in a medium containing appropriate ^{14}C- or ^{3}H-labelled compounds and the label traced in various cellular metabolic products by the use of suitable detectors. In this way the metabolism of various substrates may be conveniently studied in vitro and comparisons made with similar systems under disease states. Differences may lead to information about alteration or defects in the normal metabolic pathway in the degradation of the labelled compound under investigation. Furthermore, drug effects can be conveniently studied in vitro.

Wherever and whenever possible, by the substitution of in vitro studies using radioactive isotopes for those in vivo, any radiation exposure and inconvenience to the patient should be avoided. In addition, in vitro systems are less complex and more amenable to studies of drug effects without any risk to the individual.

Breath analysis has been used in recent years to determine the rate of $^{14}CO_2$ appearance in the breath after intravenous administration of ^{14}C-labelled compounds to the subjects. By this technique altered oxidation of the methyl carbon atom of L-methionine-methyl-^{14}C has recently been shown in schizophrenic and depressive patients.

The work presented here makes use of human leukocytes separated from whole blood of controls and patients with known disorders in establishing differences in their metabolism of appropriately labelled substrates. Thus, metabolism of uniformly ^{14}C-labelled L-asparagine by the leukocytes has been compared in chronic myelogenous leukemic patients and controls. Also, in the leukocytes from schizophrenic patients an alteration in the oxidation of the methyl carbon atom of L-methionine has been established.

II. LEUKOCYTE METABOLISM AND EXPERIMENTAL METHODS

A. General Survey

For many decades leukocytes have been subjected to various metabolic, biochemical, and histochemical tests. As early as 1911 existence of oxidative metabolism in human leukemic leukocytes was demonstrated by Grafe.[1] In the same year Slosse observed lactic acid formation in blood from glucose.[2] In 1912 glycolytic activity in leukocytes prepared from sterile exudate was reported.[3] Warburg, in 1926, established that malignant neoplasms elaborate significant amounts of lactic acid aerobically, i.e., in the presence of oxygen. Following these findings, the literature was flooded with reports of numerous comparative assays in an attempt to establish differences between metabolism of leukocytes from normal individuals and those from patients. For a long time attention was directed to various types of leukemia with regard to glucose metabolism. A critical survey of methods used until 1938 in metabolic studies was presented by Kempner.[4] Most of the experiments of that era appear to suffer from lack of satisfactory isolation methods and from imposed unphysiological conditions. General findings of most them, however, indicate the rate of glycolysis of normal white cells to be significantly higher than oxidative metabolism and lactic acid to be formed aerobically. This observation was amply documented later.[5] Numerous later attempts, as well, to demonstrate possible differences between glucose metabolism of normal and leukemic cells, have not led to sufficiently conclusive quantitative results to warrant the use of such a criterion as an indicator for a specific type of leukemia. More recently investigators have turned to utilization of substrates other than

glucose in comparative metabolic studies with leukocytes.

In 1950 incorporation of ^{14}C-leucine and ^{14}C-alanine into white cell proteins was found to be six times greater in acute leukemic cells than normal leukocytes.[6]

Weisberger and his co-workers reported in 1954 that white cells in both acute and myelogenous leukemia utilize in vivo exogenous ^{35}S-L-cystine and ^{35}S-L-methionine at a faster rate than normal leukocytes.[7] In vitro also, leukemic leukocytes exhibited higher uptake of these amino acids than the normal ones.[8]

A few years later it was found that both normal and leukemic leukocytes were capable of incorporating ^{14}C-leucine into their proteins in vitro.[9] Cells from patients with myelogenous leukemia were able to incorporate significantly greater amounts of this amino acid and also maintain the incorporation for a longer period of time than the control cells.

Attention was then turned to studying the effect of some anti-tumor drugs on amino acid incorporation into leukocytes. In 1959 an attempt was made to find out whether incorporation of formate-^{14}C and glycine-1-^{14}C could be used for screening potential chemotherapeutic agents for treatment of leukemia.[10] Unfortunately, with the drugs tested, correlation between clinical and in vitro effects could not be established.

Later in 1961, Nadler and his group showed that labelled valine and leucine were incorporated at a higher rate by leukocytes of patients with chronic granulocytic leukemia than by leukocytes of normal subjects.[11] Furthermore, they reported that it was only a few days after therapy with 6-mercaptopurine that leukocytes exhibited significant

decrease of incorporation of these amino acids, and suggested this as a possible reason for the failure to observe the effect of anti-tumor drugs by Winzler and his collaborators.

The more recent observation that patients with leukemia had an increased level of methionine in their urine revived interest in comparative assay of methionine metabolism of normal and leukemic leukocytes in vitro.[12] Sloane and her co-worker assayed incorporation of ^{35}S-labelled methionine into protein of normal and various leukemic leukocytes.[13] Although methionine uptake in case of acute leukemias and chronic myeloid leukemias was found to exhibit a range wider than in the case of control individuals, no correlation could be obtained between methionine uptake and peripheral counts.

It is unfortunate that in most of these earlier studies leukocytes have been considered as a homogeneous population, and differences in metabolism of various cell types present have been disregarded.

In spite of voluminous work done in search for exploitable metabolic differences between normal and leukemic cells, no conclusive results have yet been reached. There may very well be a general increase in incorporation of most amino acids into proteins in leukemic cells. If, however, an amino acid can be found to be absolutely essential for the survival of a particular leukemic cell type, this finding could be exploited in treatment or perhaps cure of leukemia. Depletion of the system of this amino acid by using the right enzyme, for instance, might offset changes characteristic of the leukemic cells.

Studies with leukocyte metabolism have, however, been more fertile in investigations of inborn errors of metabolism. With

the availability of improved techniques in their separation from whole blood, white cells gained popularity for use in studies of metabolic diseases during the past decade. Investigators have made use of leukocyte metabolism to localize the metabolic block in methylmalonic aciduria and show its dependence on B_{12},[14] to demonstrate a defect in pyruvate decarboxylase,[15] to study the differences in glycine-^{14}C incorporation in nucleic acid purine by leukocytes from gouty patients,[16] to analyse correction of metabolic deficiencies in leukocytes from patients with chronic granulomatous disease of childhood,[17] and to investigate defective propionate carboxylation in ketotic hyperglycemia.[18] A plethora of metabolic diseases have been analysed by this method. The pathological states studied up to 1969 are thoroughly discussed by Hsia.[19]

Various aspects of metabolism of leukocytes are reviewed in an excellent article by Cline.[20] Metabolic patterns of normal and abnormal cells, though morphologically similar, may differ appreciably. Finding the altered patterns and correlating them to the biological state may lead to understanding the etiology of the disorder, and may give a clue about therapy to be used in correcting the altered pattern. In general, changes in incorporation of suitable substrates into cellular proteins and/or alterations in their oxidation rates may be studied in vitro.

B. Choice of Leukocytes for the Assays and Technique of Their Isolation from Whole Blood

The easy availability of leukocytes from peripheral blood and their relative resistance to unphysiological conditions during isolation procedures favour their choice for in vitro studies. Their endowment with major cell organelles renders them suitable for assays of oxidative metabolism as well. In the past the major problem in their use for

clinical investigations in vitro, however, rested in the lack of satisfactory techniques for their isolation from other blood elements in adequate quantities, uninjured and completely viable. For the last decade or so better methods have become available. The advent of the electron microscope has been invaluable in revealing changes in the morphology of their subcellular organelles when subjected to prolonged unphysiological conditions in the process of separation and in assessing optimal conditions for their viability.

Naturally, any study of leukocyte metabolism in vitro necessitates application of the best techniques available for their separation from whole blood. A detailed account of the wide spectrum of techniques used until now is given elsewhere.[21]

To render blood uncoagulable, heparin has been chosen as an anticoagulant. Leukocytes separated from blood when heparin is used as anticoagulant appear to be normal by their ameboid motility and biochemical tests as observed by several experimenters. Heparin used in the following experiments was free of phenol, and had 0.9% benzyl alcohol as preservative.

Blood once rendered uncoagulable has to be subjected to certain procedures for isolation of viable leukocytes. The fact that many methods have been described for white cell separation simply indicates that no one method alone has been completely successful. From the methods used for this purpose, differential sedimentation followed by selective RBC destruction has been chosen for the present study.

Density difference between formed elements of blood has long been used in achieving partial isolation of these elements, the denser

erythrocytes (1.092) settling faster than leukocytes (1.065). Differential sedimentation of RBC's is found to be enhanced by erythrocyte rouleaux formation. This is achieved by using agents like phytohemagglutinin and some plasma expanders such as dextran, the latter being in more popular use. Skoog and Beck made a beautiful comparative assay on the use of fibrinogen, dextran, and phytohemagglutinin methods for differential sedimentation.[22] They defined certain variables to be considered for achievement of optimal isolation evaluated by yield and purity of the leukocytes. In the present work the ratio of 1:2 of 3% dextran to blood has been used.

In case of selective lysis, the agents which bring about lysis of RBC's without affecting leukocyte population are used. Distilled water is one of such agents. Exposure to hypotonicity must, however, be very short, and concentrated salt solution must be added to recover isotonicity. Fallon and his co-workers studied the effect of hypotonicity on leukocyte morphology.[23] Extensive damage in the majority of cells and severe fragmentation in many resulted when cells were subjected to hypotonicity for four minutes. After exposure to hypotonic saline (0.22%) for 30 seconds, however, less than 5% of leukocytes were found to reveal damage when examined under electron microscope.

C. Experimental Methods

1. Method of Isolation of WBC's from Whole Blood

Venous blood was drawn into a plastic syringe previously heparinized with lipo-hepin (Riker Laboratories) containing 0.9% benzyl alcohol as a preservative. RBC's were allowed to sediment differentially from a mixture of whole blood and 3% dextran in saline (w/v), in the ratio of

2:1 respectively for about 30 min. after thorough mixing by gently inverting the tube containing the mixture seven times. The supernate containing leukocytes, platelets, and variable amounts of erythrocytes and plasma was centrifuged in 40 ml cones at 100 g. The supernatant fluid was discarded by suction, and the cell button was suspended in 20% fetal calf serum and 10% Hanks' solution and washed with 20 ml of this preparation. After centriguation, the cells were washed for a second time and further centrifuged. For elimination of RBC's, they were subjected to hypotonic lysis. At the end of the second washing the cell button was suspended in 4 ml physiological saline (0.9% NaCl), and 12 ml sterile distilled water was added to bring the percentage of NaCl to 0.22. To help lysis of the swollen RBC's, mechanical agitation was applied by drawing the mixture rapidly up and down a 10 ml serological pipette. This whole procedure was limited to less than 30 sec. to minimize any change of WBC morphology which is reported to occur upon prolonged exposure to hypotonicity. Isotonicity was restored by the addition of 4 ml of 3.5% NaCl. Five ml of minimum essential medium (MEM) was added before centrifuging it for the final time. The red cell ghosts were removed from the surface of the cell button, which was resuspended in MEM and diluted to the desired degree.

Each centrigation lasted for 4 to 7 min. All the glassware was siliconized and sterilized by autoclaving. Cell counts on the separated leukocytes were done in duplicate by a hemocytometer in the standard way described elsewhere.[24] RBC and WBC counts on whole blood were done using a Coulter counter. Differential WBC counts were carried out on both whole blood and the isolated cells. Separated cells were

stained by Wright's stain. The final suspension contained practically no RBC, and consisted of granulocytes in the range of 80 to 90%.

2. Evidence for Functional Integrity of Leukocytes Following Isolation

Leukocytes prepared as described above were tested for cell viability by currently available criteria after the isolation process. They appeared normal under the light microscope: they formed pseudopodia and possessed ameboid motility, excluded trypan blue or eosin dyes,[25,26] phagocytized latex particles, and metabolized glucose and amino acids to CO_2. Satisfaction of these criteria was interpreted as an indication of the metabolic integrity of the leukocytes. At the end of an incubation period of a few hours, they were tested for viability by the first three criteria mentioned above, and appeared to retain it.

It may be briefly mentioned that a dye, such as trypan blue or eosin Y, is commonly used in the determination of cell viability. A certain concentration of dye, in this case 0.05% trypan blue or eosin Y, is mixed with some cells and viewed under the light microscope. Stained cells are assumed to be damaged, and the unstained ones are considered to be undamaged. Most of the cells that do take up the dye are found to be grossly swollen but yet retain their general appearance.

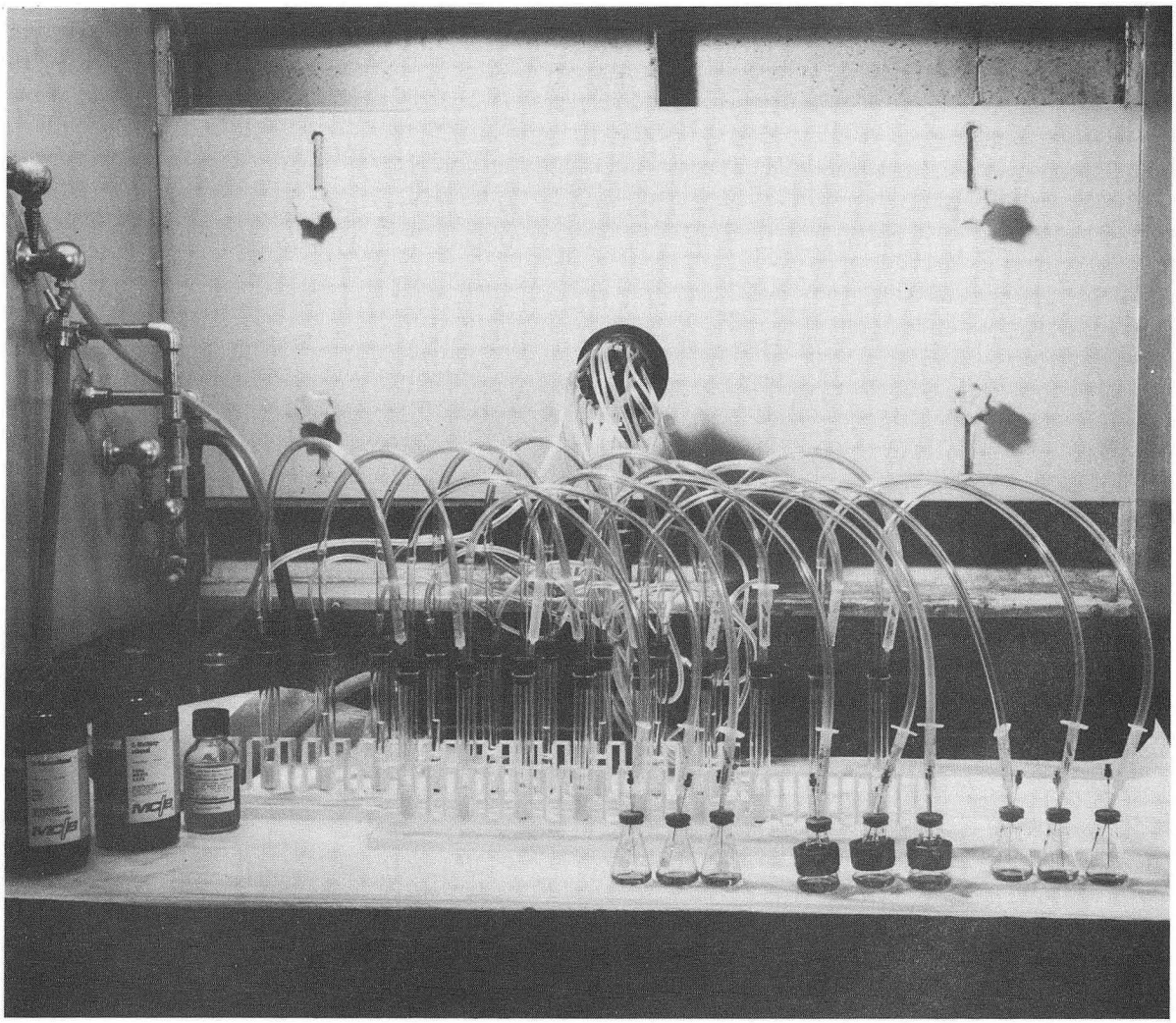
For phagocytosis, latex particles of approximately 1μ in diameter were used.

3. Incubation of Cells and Collection of CO_2

One ml aliquots of cell suspension in MEM, containing known numbers of cells as determined by hemocytometer counts were transferred into 25 ml Erlenmeyer flasks, each containing 2 ml MEM—the

nutrient medium. Following a preincubation of 30 to 40 min at 37°C, labelled substrate was injected into each flask topped with an airtight rubber cap. For estimation of substrate background due to decomposed substrate, the same amounts of labelled material were introduced in similar flasks containing equal volumes of the medium. The flasks were kept in the water bath at 37°C and mild agitation (50 strokes per min.) was applied. CO_2 produced as a result of metabolism of the labelled compound by the cells was allowed to accumulate in the flasks. At the end of the desired incubation period, 2 ml of 2 M acetate buffer at pH = 3.8[27] was injected into each flask to terminate metabolic activity of the cells and also to release CO_2 that might be present in bicarbonate form. The efficiency of CO_2 release by the addition of the acetate buffer was tested by adding a known amount of ^{14}C-labelled sodium bicarbonate to 3 ml MEM and driving CO_2 off with 2 ml of the acetate buffer. The quantity of CO_2, trapped by the absorbing mixture, was found to be equal to the activity initially present in the added sodium bicarbonate.

To trap CO_2, an arrangement shown in Fig. 1 was used. Roughly 8.5 ml of a mixture of 2-aminoethanol and 2-methoxyethanol was dispensed into preweighed 40 ml test tubes fitted with rubber stoppers having a central bore through which a disperser tube could be inserted. The bubbler end of the disperser dipped into the mixture. Vacuum was applied to the space above the CO_2-absorbing fluid. A continuous air flow through the flasks was maintained by using a 22-gauge needle. Air was bubbled through the liquid in the flasks. Excessive bubble formation was prevented by the addition of small amounts of a solution of anti-foam



XBB 726-3464

Fig. 1.

to each flask. To ensure that a significant amount of CO_2 was not lost, a second tube was connected in series with the first one. The collection of CO_2 was carried out overnight. Background levels of $^{14}CO_2$ were assessed in a similar way. At the end of the collection period the tubes were reweighed and 4 ml aliquots of CO_2-trapping fluid were placed in counting vials and weighed. To each vial 15 ml of scintillation fluid consisting of a mixture of toluene, methoxyethanol, and PPO (2,5-diphenyloxazole) was added, and ^{14}C activity was counted in a Nuclear Chicago liquid scintillation counter.

4. Assessment of Total Activity Retained by Cells

To determine the total radioactivity fixed by the cells, additional samples were incubated as described previously. At the end of the incubation period metabolic activity was terminated by adding 5 ml of ice cold saline to the cell suspension and placing the flask in ice cold water. CO_2 collection from space above the cells was carried out for 5 min. The cells were then centrifuged at 0°C and the supernate decanted. The cells were washed three times with cold saline (0°C). At the end of the washing procedure, 1 ml of NCS, a strongly alkaline toluene solution, was added to the cells to solubilize them. After the digestion by NCS the digest was transferred into counting vials. To each vial 15 ml of scintillation fluid, described above, was added before counting in a liquid scintillation counter.

5. Determination of Protein Moiety of the Cellular Activity

Further additional samples were incubated for measurement of incorporation of labelled substrate into cellular proteins. The reaction was terminated, and the cells were washed as in the case of assessment

of total cellular activity. One ml of 15% TCA (ice cold) was added to each sample and allowed to stand for 30 min. in ice cold water. The precipitate was centrifuged and washed in 1 ml of ice cold 15% TCA. A second wash with 1 ml of ice cold absolute alcohol was followed by the addition of 1 ml NCS to digest the protein.[28] The digest was then counted for its activity after the addition of 15 ml of the scintillation fluid.

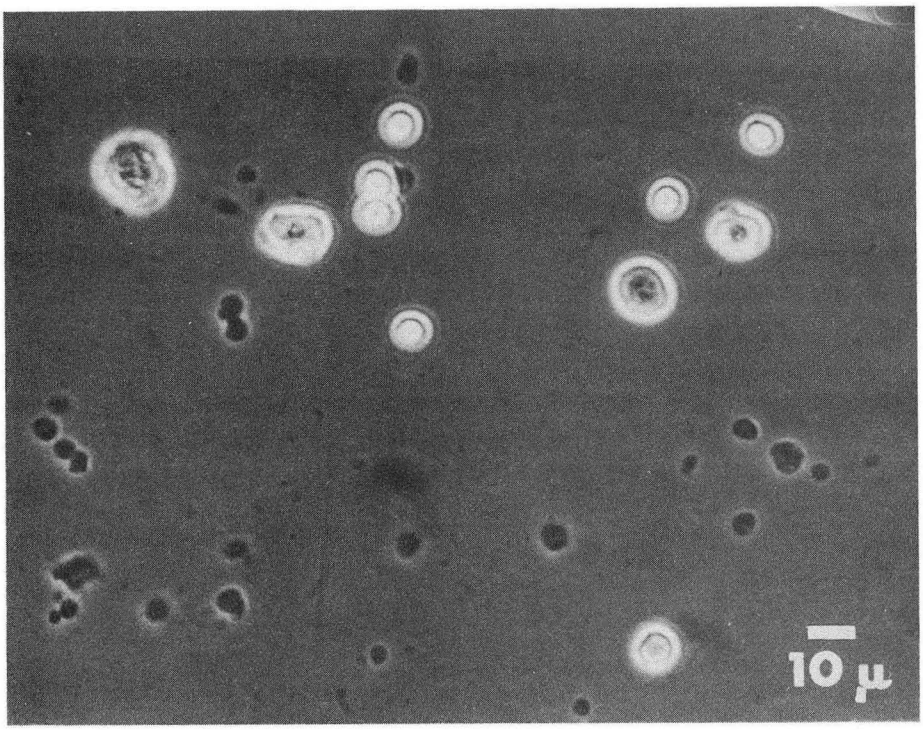


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Fig. 2. In a phase contrast photomicrograph, the appearance of the leukocytes isolated by Skoog and Beck's dextran method before the lysis of the residual red blood cells. Six red cells and five leukocytes (four large ones and a smaller one at the lower right quarter of the photograph) are shown suspended in a solution of 0.05% trypan blue. Note that these viable cells have excluded the dye. Cells which take up the dye appear dark under phase.

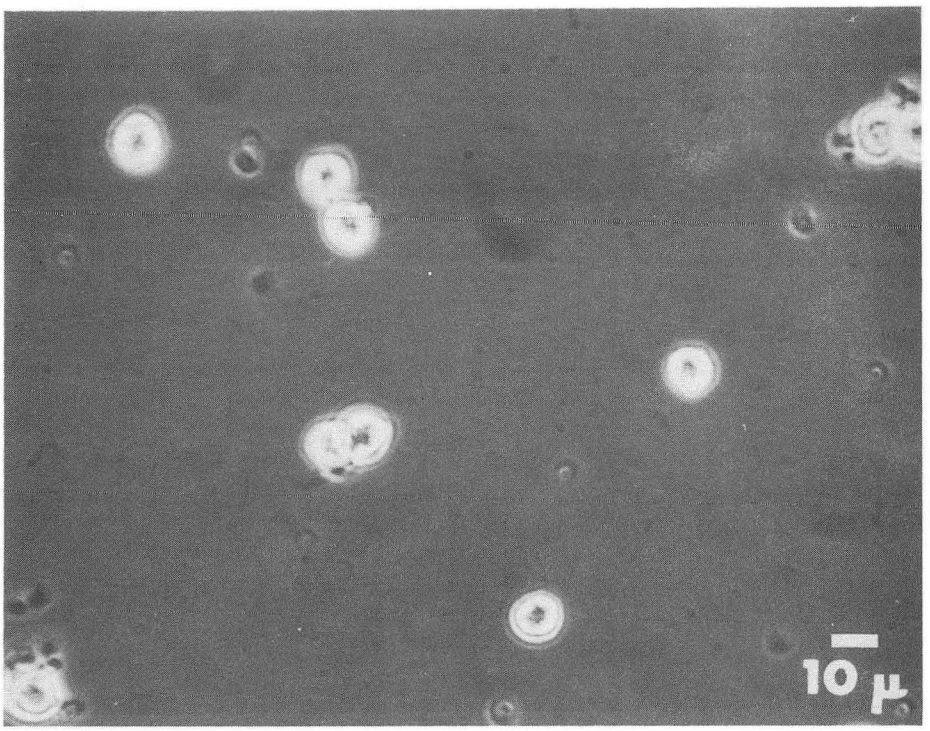

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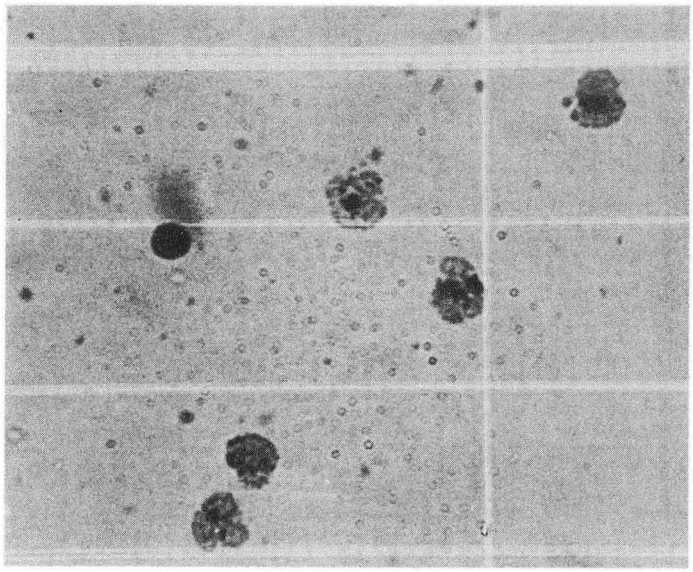
Fig. 3. A phase contrast photomicrograph of the isolated leukocytes in a solution of 0.05% trypan blue after hypotonic lysis of the residual red blood cells, following separation by dextran-blood sedimentation. It should be noted that all these cells are leukocytes and that they have all excluded the dye.



XBB 727-3716

Fig. 4. The appearance of dead leukocytes in a solution of 0.05% trypan blue in a hemocytometer. The cells were killed by a toxic dose of chlorpromazine. Note that these dead cells have all taken up dye and appear dark in this photomicrograph.

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III. METABOLISM OF UL-^{14}C-GLUCOSE AND GLUCOSE-1-^{14}C, AND OXIDATION OF L-METHIONINE-METHYL-^{14}C TO $^{14}CO_2$ BY HUMAN LEUKOCYTES IN VITRO

A. Summary

To assess the metabolic competence of the isolated leukocytes, their metabolism of UL-^{14}C-glucose, glucose-1-^{14}C, and L-methionine-methyl-^{14}C to $^{14}CO_2$ was investigated. The rates of $^{14}CO_2$ production from these compounds, as measured by the disintegrations per minute per 10^8 cells per hour per μCi of tracer were found to be constant for each subject after the tracer equilibrated in the system. The constancy of these rates persisted for a few hours, indicating that the separated leukocytes retained their initial ability to metabolize glucose and oxidize the methyl carbon of L-methionine to CO_2 for the period of time during which the experiments were carried out.

B. Introduction

Grafe was first to demonstrate the existence of oxidative metabolism in leukemic human leukocytes.[1] Later work showed that they possess Krebs' cycle activity.[2] It is known that leukocytes may lose their functional integrity in the following descending temporal order: oxidative metabolism, phagocytic activity, ameboid motility, Brownian movement, resistance to impermeable dyes, and finally morphologic integrity.[3] This suggests that an assay of the oxidation of an amino acid such as L-methionine will be most valuable as an indicator of the retention of the metabolic competence of the cells subjected to in vitro separation procedures.

C. Materials and Methods

UL-^{14}C-glucose (specific activity 3mCi/mM, New England Nuclear Corp.) and glucose-1-^{14}C (specific activity 6.3 mCi/mM, New England Nuclear Corp.) were used for the glucose assays. L-methionine-methyl-^{14}C (specific activity 11 mCi/mM) was also obtained from New England Nuclear Corp.

Leukocytes separated from fresh blood of control subjects, using the technique described in the previous section, were suspended in 3ml MEM containing glucose at a concentration of 1 mg/ml. MEM used for L-methionine assay was methionine-free. About 1 μCi of labelled substrate was administered to each flask holding the cells of the sample, after a preincubation period of 40 min. Cell number was allowed to vary in the range of $(1.4\text{-}2.3)10^7$ from one subject to the other. At the end of the desired incubation period, the metabolic reaction was stopped and the CO_2 liberated was collected in the way described in Section II.

With labelled glucose, two series of experiments were carried out:

1) To establish the nature of variability of the amount of $^{14}CO_2$ production with the number of cells, leukocytes whose number varied in the ratio 2:3:4:5 were used in 3 ml MEM and the radioactivity of each sample was kept constant. Cell number was varied in the range of $(1\text{-}2.5)10^7$ for various samples which were incubated for the same length of time. A plot of the radioactivity from $^{14}CO_2$ collected against the ratio of cell number in the medium was obtained.

2) To investigate the relationship of the amount of CO_2 production to the time of incubation, the cell number in each sample obtained from

the same person was held constant and the period of incubation was varied from one sample to the other. Duplicate samples were incubated for each measurement, and radioactive counts were obtained by using the scintillation counter. Counting efficiencies as determined by internal standardization with standard toluene ^{14}C solution (7.15×10^5 dpm/g from Amersham/Searle) were in the range of 53.6 to 55.0%. Counter background varied in the range of 20-28 counts per minute. The samples were counted for a time sufficiently long to give an accuracy better than 2.5%. Disintegrations per minute were obtained by multiplying total counts per minute by the efficiency factor of detection. Dpm's were normalized for 10^8 cells/μCi. The oxidation rates were expressed as dpm/10^8 cells/hr/μCi. This latter value was used in calculating the fraction of radioactivity going into $^{14}CO_2$. Knowledge of the amount of glucose initially present in the medium provides a means of calculating μM of glucose metabolized to CO_2 from the one-position carbon atom of glucose-1-^{14}C, with the assumption that during the incubation period there is no appreciable change in the glucose concentration of the medium in which the cells are suspended.

D. Results

Figures 1 and 2 show the radioactivity in the $^{14}CO_2$ produced from the metabolism of UL-^{14}C-glucose as a function of time in two different subjects. After an initial lag time the rates of $^{14}CO_2$ production appear to be constant well beyond two hours.

The relationship between $^{14}CO_2$ liberated from the uniformly ^{14}C-labelled glucose metabolized by the cells of varying numbers incubated in equal volumes of medium for the same period of time is shown in

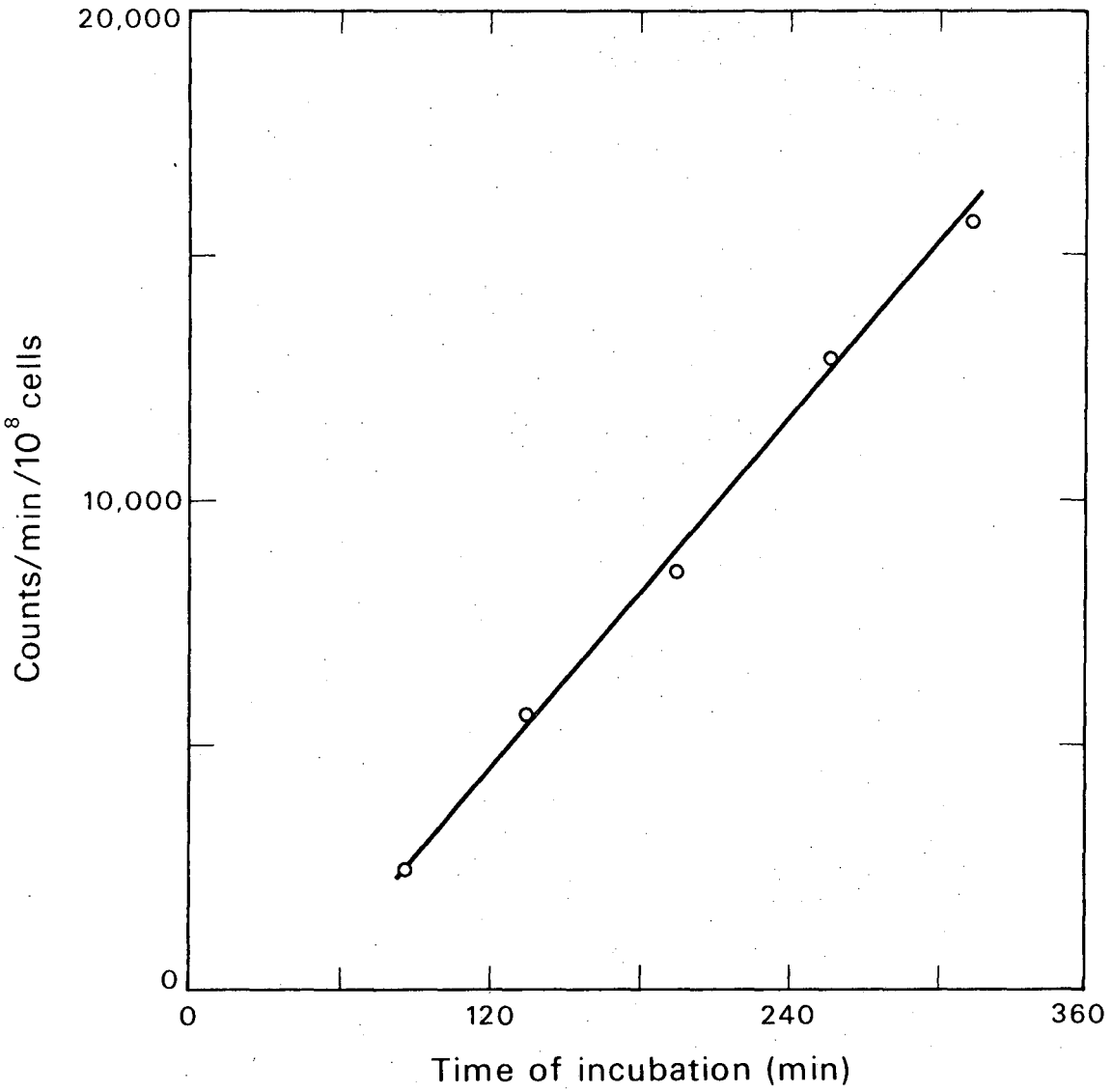

Fig. 1. $^{14}CO_2$ production as a result of metabolism of UL-^{14}C-glucose by human leukocytes. The graph depicts the counts/min normalized for 10^8 cells from samples containing cells in 3 ml buffered MEM and 1 μCi of UL-^{14}C-glucose. Note that the cells of this subject appear to retain their initial activity of metabolizing glucose more than 3 hours.

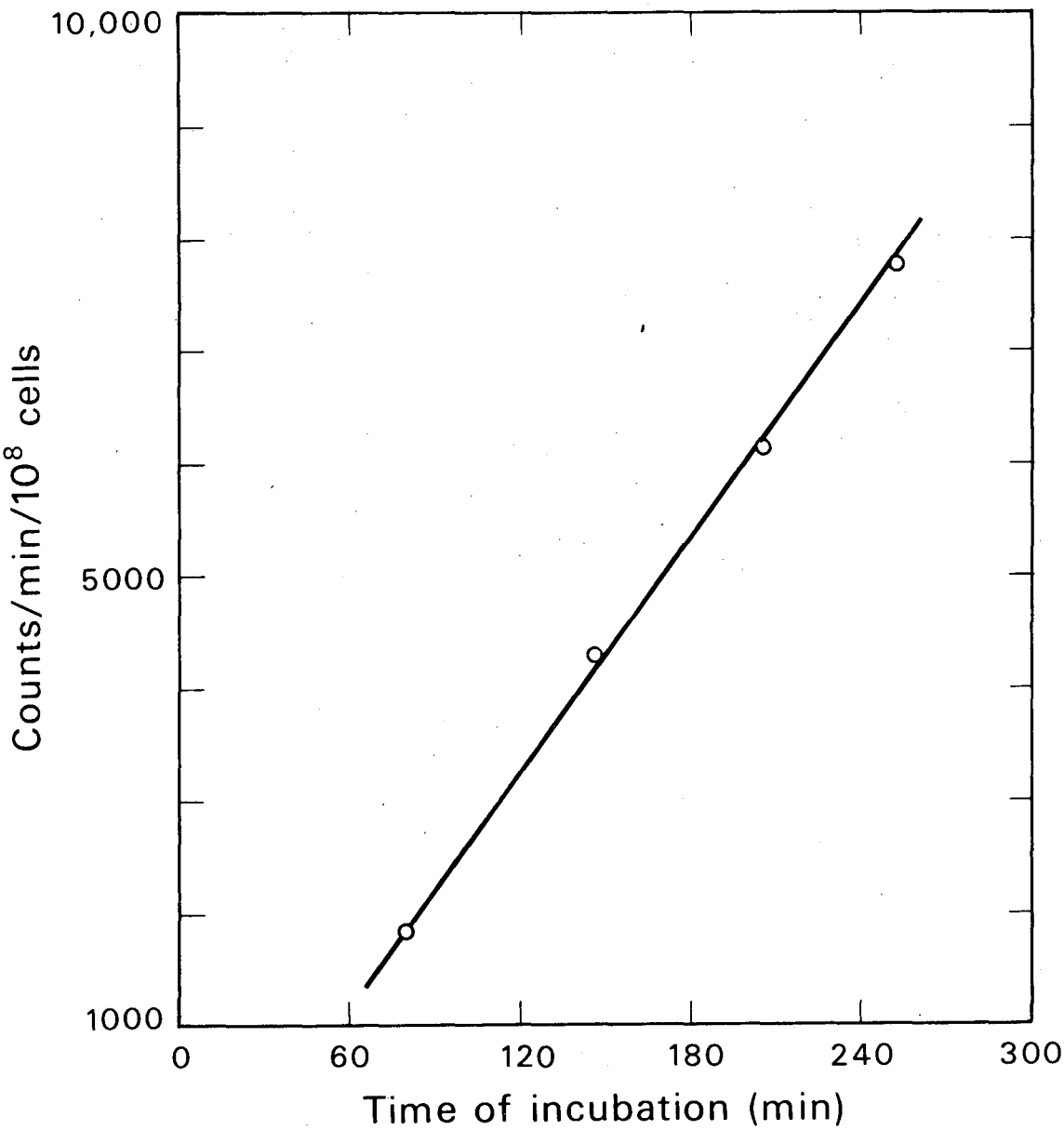

Fig. 2. $^{14}CO_2$ production as a result of metabolism of UL-^{14}C-glucose by the leukocytes of another subject.

Fig. 3.

Figure 4 illustrates a typical curve from which dpm/10^8 cells/hr/μCi was obtained in calculating the data in Tables 1 and 2. These tables summarize some metabolic data obtained for UL-^{14}C-glucose and glucose-1-^{14}C. The fraction of glucose metabolized to $^{14}CO_2$ from all six carbon atoms per 10^8 cells/hr was calculated to be $(2.8 \pm 0.3)10^{-3}$ when 1 μCi of UL-^{14}C-glucose was used as tracer. The fraction of 1 μCi of glucose-1-^{14}C metabolized to $^{14}CO_2$ from one-position carbon atoms per 10^8 cells per hour was found to be $(5.6 \pm 0.6)10^{-3}$.

Figure 5 depicts $^{14}CO_2$ production by the leukocytes from a depressive subject under drug therapy after the administration of 1 μCi of L-methionine-methyl-^{14}C to the cells suspended in a methionine-free medium.

E. Discussion

The straight-line nature of the curves depicted in Figs. 1 and 2 indicate the retention of initial viability of the cell population with respect to the ability of the cells to metabolize glucose to CO_2. It is reasonable to expect that the initial rate of metabolism of the separated cells will remain uniform for a period of time well beyond the maximum two hours' incubation of the experiments.

The results shown in Fig. 3 suggest that the ability of the cells to metabolize glucose does not vary when the cell concentration is in the range of $(3.3\text{-}8.4)10^6$ cells per ml.

The initial apparent lag time of the glucose experiments may be explained to be due to the time required by the tracer to reach a state of complete mixing within the glucose pool.

The analysis of the data in Tables 1 and 2 suggests that an appreciable amount of, but not all, the contribution to CO_2 production comes from the one-position carbon atom of glucose carbon chain. It is known that the one-position carbon atom of glucose-6-PO_4 entering the pentose cycle is directly metabolized to CO_2. If only the one-position carbon atom of glucose was metabolized to CO_2, the $^{14}CO_2$ produced when 1 μCi of uniformly ^{14}C-labelled glucose was used in place of 1 μCi of glucose labelled in the one-position carbon atom would be expected to be 1/6 of that for the latter. However, as indicated by the results of Tables 1 and 2 this ratio is 1/2, suggesting that there is some contribution to $^{14}CO_2$ produced from the metabolism of carbon atoms in the position other than one, through a metabolic pathway different from the pentose shunt.

The constancy of the rate of oxidation of the methyl carbon of L-methionine-methyl-^{14}C is substantial evidence in favour of the retention of the viability with regard to oxidative metabolism of the cells subjected to the isolation procedures.

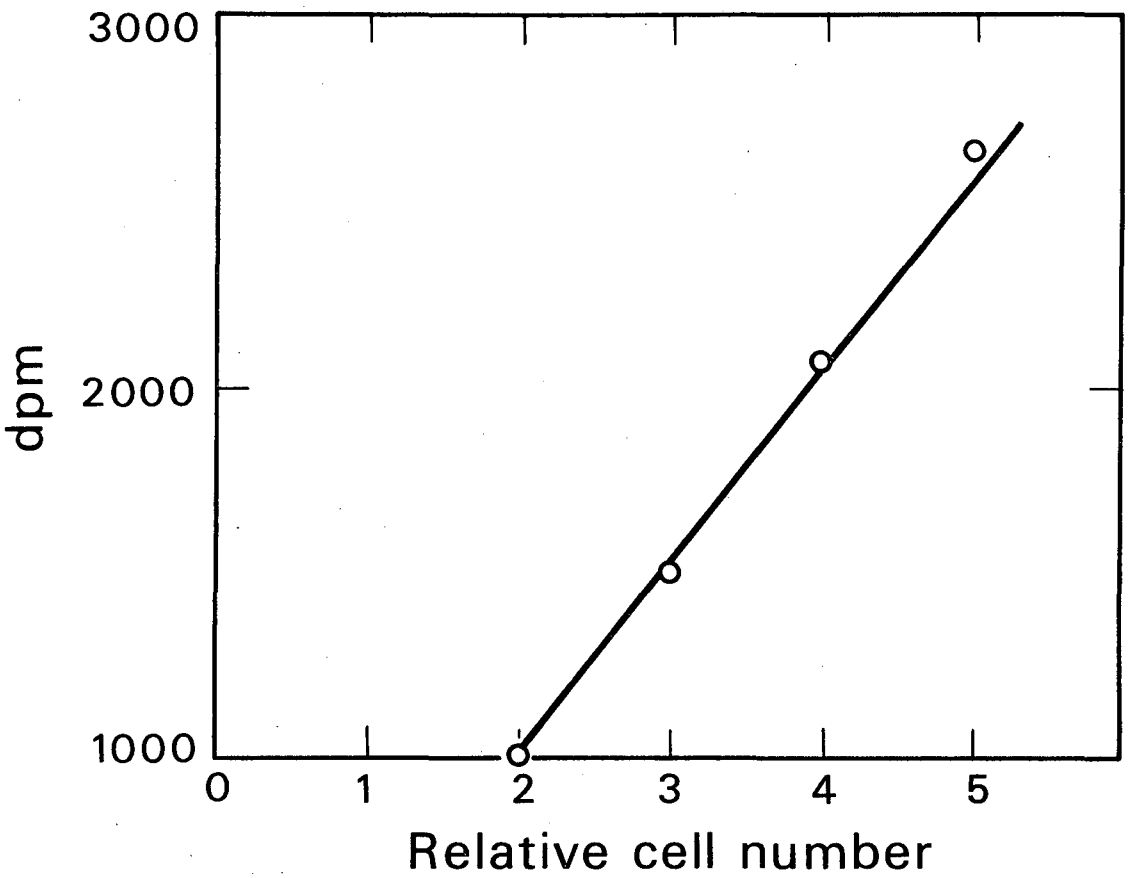

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Fig. 3. The effect of cell concentration on the $^{14}CO_2$ production from UL-^{14}C-glucose by human leukocytes. The graph shows the radioactivity in $^{14}CO_2$ liberated by the cells of differing concentration in 3 ml buffered MEM. The incubation period in this instance was 2 1/2 hours. The linear nature of this curve should be noted.

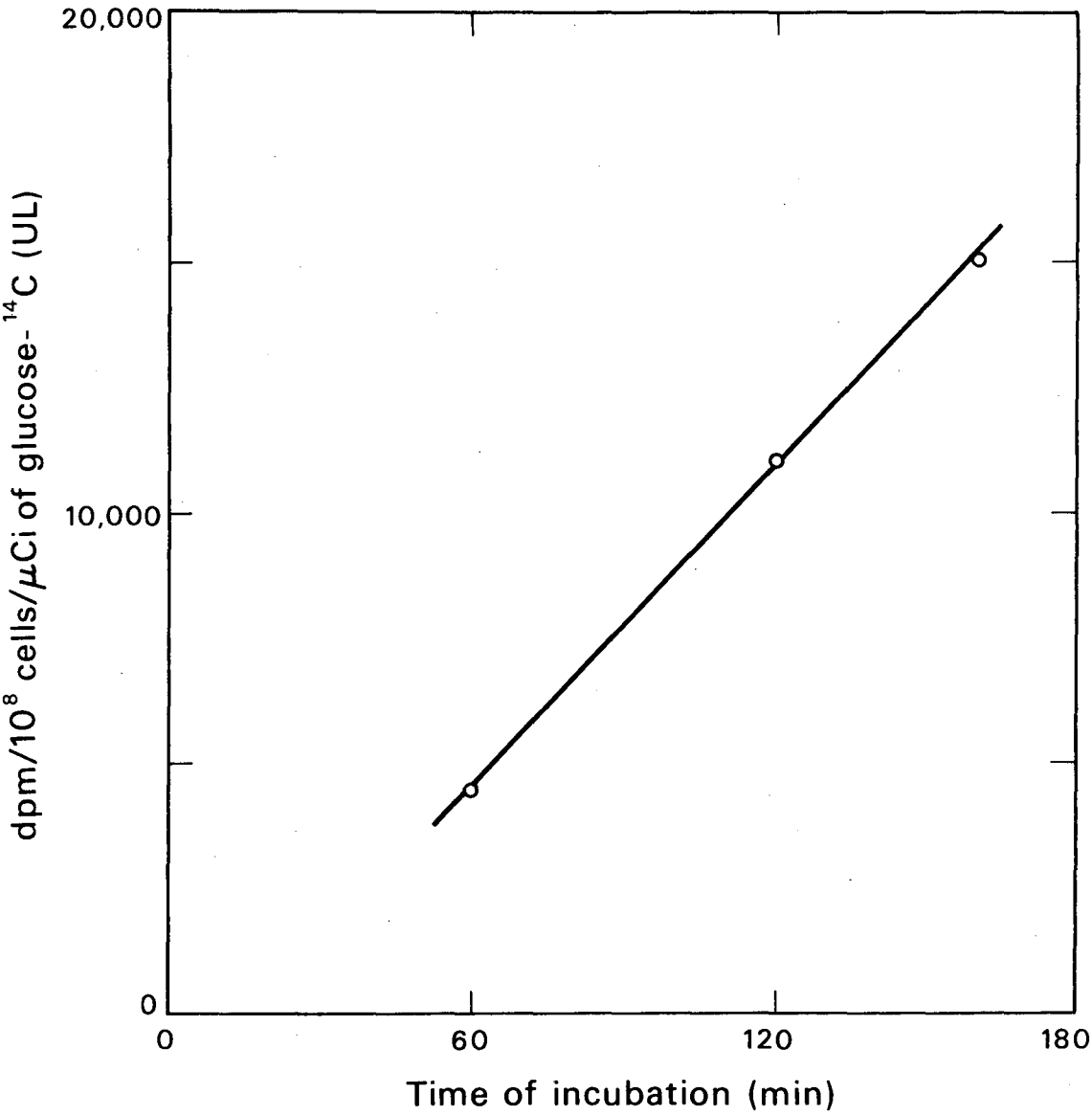

Fig. 4. This graph illustrates a typical curve from which the rate of metabolism of UL-^{14}C-glucose to $^{14}CO_2$ was obtained as dpm/10^8 cells/hr/μCi.

Table 1. Metabolism of UL-^{14}C-glucose to $^{14}CO_2$

Subject	Fraction of 1 μCi UL-^{14}C-glucose metabolized to $^{14}CO_2$/10^8 cells/hr from all carbon atoms ($\times 10^{-3}$)
1	2.9
2	2.7
3	2.9

Table 2. Metabolism of glucose-1-^{14}C to $^{14}CO_2$

Subject	Fraction of 1 μCi of glucose-1-^{14}C metabolized/ 10^8 cells/hr to $^{14}CO_2$ from carbon atoms at one position ($\times 10^{-3}$)
1	4.9
4	5.6
5	6.3

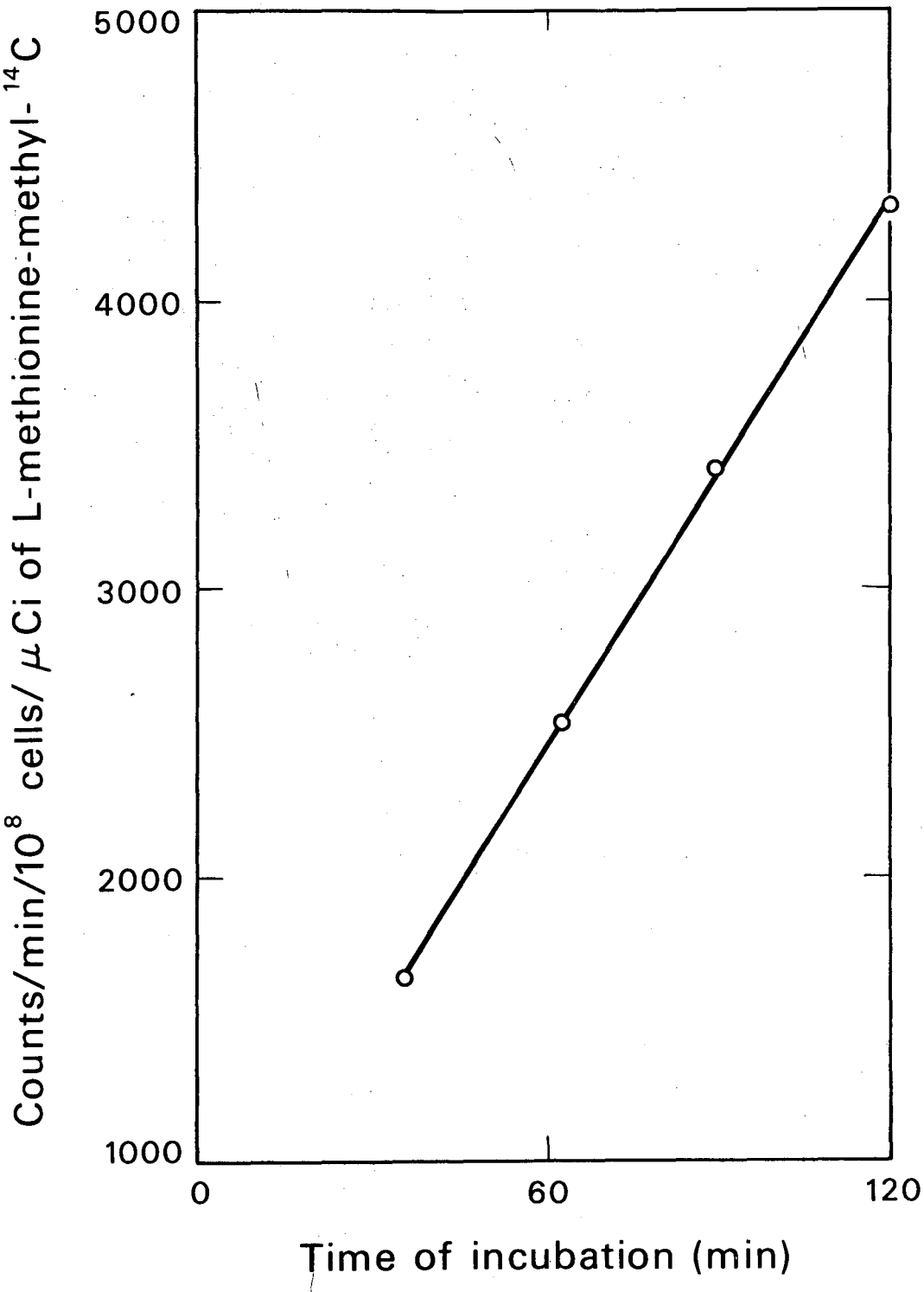

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Fig. 5. Oxidation of methyl carbon atom of L-methionine-methyl ^{14}C by the leukocytes of a depressive patient under drug therapy is shown in this figure. Initially there was no methionine in the incubation medium. 1μCi L-methionine-methyl-^{14}C was added to the cell suspension.

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IV. L-ASPARAGINE METABOLISM OF LEUKOCYTES FROM PATIENTS WITH CHRONIC MYELOGENOUS LEUKEMIA

A. Summary

Some aspects of metabolism of L-asparagine-^{14}C-(UL) were studied in vitro in leukocytes consisting mostly of granulocytes separated from venous blood obtained from subjects with normal hemograms and patients with chronic myelogenous leukemia (CML). The rates of cellular uptake of this amino acid, its incorporation into proteins, and the rate of respiratory $^{14}CO_2$ production resulting from its oxidation were investigated. Significant differences were observed between controls and leukemic subjects, both in cellular uptake and in incorporation into proteins of L-asparagine. These differences could not be abolished by preincubating the cells in a medium containing cold L-asparagine. The independence of the chronic myelogenous leukemic leukocytes of L-asparagine was demonstrated by the finding that the amount of incorporation of L-valine-^{3}H(G) into cellular proteins did not differ significantly in the presence or absence of L-asparagine in the incubation medium.

B. Introduction

The observation that guinea pig serum suppresses some transplanted lymphomas in mice and rats in vivo[1] and characterization of the active factor as protein[2] were followed by its identification as L-asparaginase.[3] This enzyme, with a molecular weight of 1.38×10^5, is known to catalyze the deamination reaction of L-asparagine.[4] Active forms have been obtained from the serum of guinea pigs[1,2,3,5,6,7] and closely related rodents,[8] as well as E. coli[9] and other bacteria.[10] Normal

tissues have been found to be temporarily affected by L-asparaginase as in the case of brief inhibition of liver regeneration,[11] but no permanent toxicity or development of serious hypersensitivity has yet been reported. However, cells seem to differ in sensitivity to this enzyme.[1] Certain lines of malignant cells require L-asparagine for growth. L-asparaginase sensitive cells of lymphoma 6C3HED have been found to be L-asparagine dependent. Resistant variants, on the other hand, are independent of this amino acid.[12] In asparagine-dependent cells, deprived of L-asparagine, which is a constituent of most proteins, protein synthesis (as measured by incorporation of exogenous valine) is inhibited.[13] L-asparaginase has also been shown to inhibit growth of some tumors, and has been used in the treatment of certain leukemias.[14,15,16] With the use of a more rapid and sensitive method of L-asparagine determination in body fluids,[17] it has recently been reported that when administered in vivo, L-asparaginase depletes plasma of L-asparagine. If, indeed, L-asparaginase suppresses certain lymphomas by diminishing availability of L-asparagine to the cells and inhibiting certain protein formation essential for the survival of leukemic cells, a comparative study of the metabolism of L-asparagine by normal and leukemic leukocytes may be valuable in predicting sensitivity of leukemic cells to L-asparaginase treatment. In the present work we have tested chronic myelogenous leukemic leukocytes for their possible dependence on L-asparagine. We have also investigated a few aspects of the metabolism of L-asparagine, namely, its cellular uptake, incorporation into proteins, and conversion into respiratory CO_2 by normal leukocytes and those from CML patients.

C. Materials and Methods

L-Asparagine-^{14}C(UL) was obtained from New England Nuclear Corp. and had a specific activity of 161 mCi/mM.

Leukocytes from venous blood drawn from healthy individuals and CML patients were isolated as described in Section II. Differential counts done on the isolated cells revealed that the final cell suspension contained a high percentage of granulocytes, 82-88% for controls and 82-95% for CML patients. Each sample in a 25 ml Erlenmeyer flask contained 3 ml MEM, about 2.5×10^7 cells, and approximately 0.8 μg (1 μCi) L-asparagine-^{14}C(UL). Six identically prepared samples were divided into three groups of pairs for the assays on CO_2 production, cellular uptake, and incorporation into proteins. All samples were preincubated at 37°C for 30 min. and incubated at the same temperature for 120-125 min. after addition of L-asparagine-^{14}C(UL). The methods for measurement of radioactivity retained in the cells, incorporated into proteins, and which appeared in CO_2, have already been described in Section II.

D. Dependence of the Rates of Incorporation of L-Asparagine into Cellular Proteins on Its Extracellular Concentration

With increasing L-asparagine concentrations in the medium the rate of incorporation of this amino acid into proteins is expected to increase until a maximum value is reached. At concentrations above that corresponding to this maximum rate, the rate of incorporation of label from L-asparagine-^{14}C(UL) is expected to be concentration independent and linearly related to the specific activity (mCi/mM) of the tracer in the medium. A plot of incorporation into proteins of L-asparagine-^{14}C(UL) against initial specific activity in the

extracellular medium is shown in Fig. 1. The initial linear portion of this curve suggests that at a specific activity below 10mCi/mM, corresponding to a concentration above 4.4 μg/ml, the rate of incorporation of L-asparagine exhibits constancy and the radioactivity of the tracer detected in cellular proteins becomes proportional to the initial specific activity of the incubation medium.

E. Major Metabolic Pathways of L-Asparagine

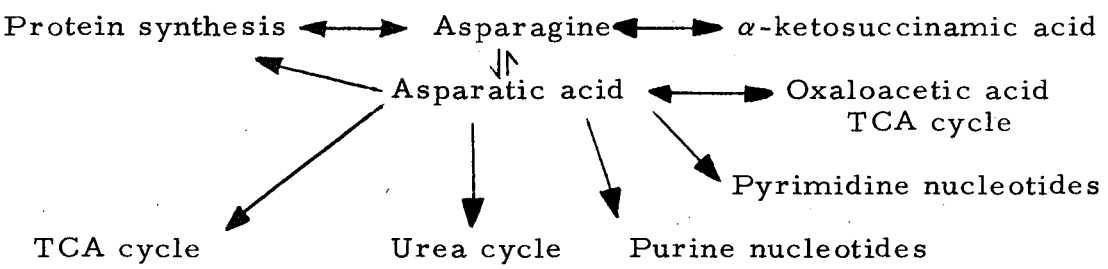

Fig. 2.

The major metabolic pathways of asparagine are shown in Fig. 2. It is known that label, detected in the cells after the uptake of L-asparagine-^{14}C(UL), is not simply due to L-asparagine-^{14}C(UL) per se, but also is due to the conversion products of this amino acid into mostly asparatic acid and to a much lesser degree into arginine, glutamic acid, and alanine.[18] These products, carrying the label from L-asparagine, may be incorporated into cellular proteins. When cellular uptake of L-asparagine increases, its labelled products are expected to do so, and as a result they cause an enhanced incorporation of the label into proteins. The results of Table 1 are consistent with this expectation.

Table 1. Metabolism* of L-asparagine-^{14}C (UL) by leukocytes from CML patients and controls.

Subject	WBC count per cu. mm in thousands	Diagnosis	Total cellular uptake		Incorporation into proteins		Oxidation to $^{14}CO_2$	
			With cold asparagine	Without cold asparagine	With cold asparagine	Without cold asparagine	With cold asparagine	Without cold asparagine
SJS	29.1	CML	22	36	7	16	3.0	1.8
CW	20.1	CML	33	49	9	29	2.0	1.0
ELW	20.2	CML	28	38	8	19	2.3	1.5
MLN	7.6	C	13	17	5	10	1.3	0.9
JMC	6.4	C	6	9	3	5	1.5	0.8
DLP	5.4	C	7	14	3	8	1.1	0.8

* Units of 10^3 dpm/10^8 cells/hr/μCi of tracer

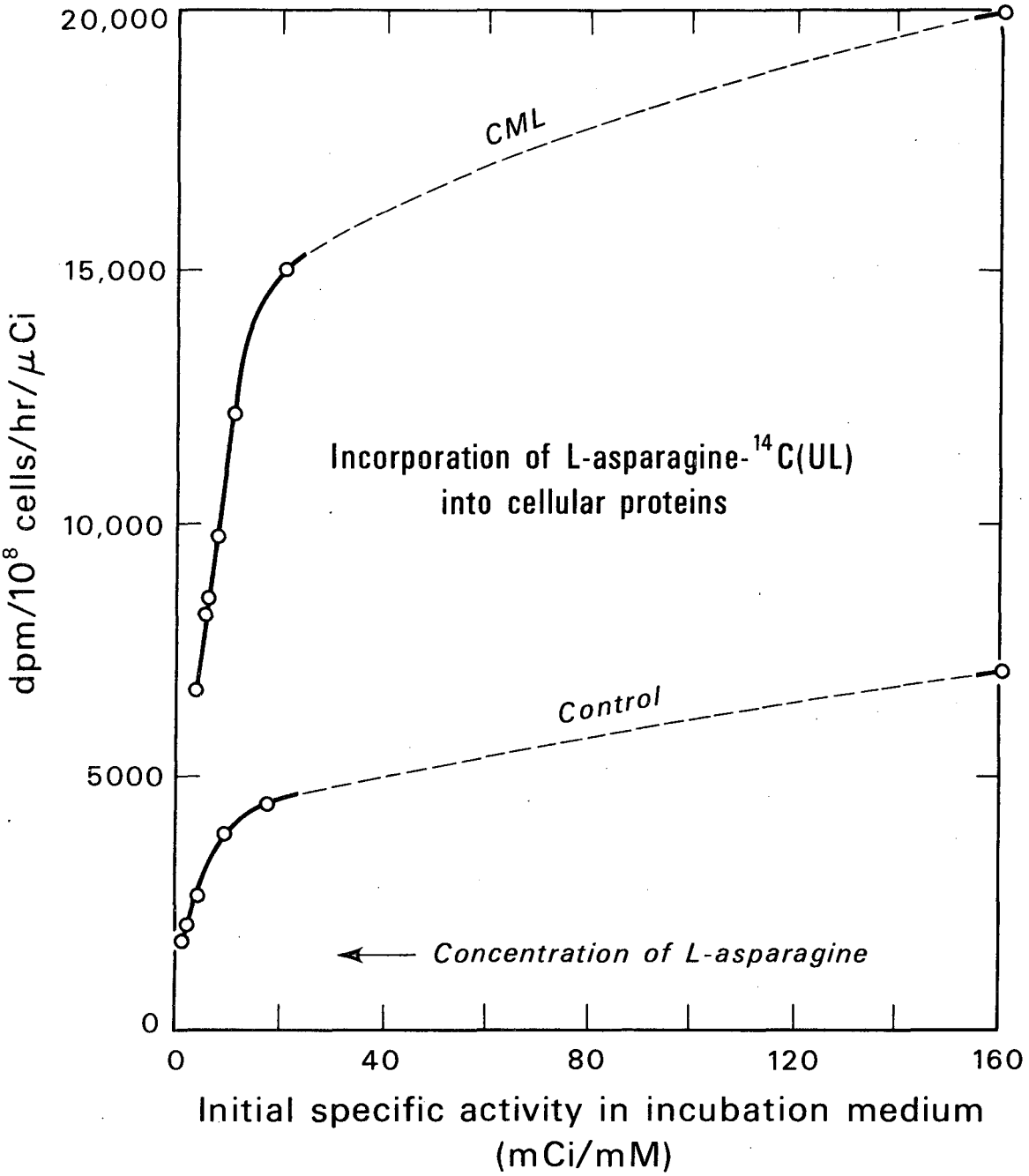
20,000
15,000
10,000
5000
0
dpm/10⁸ cells/hr/μCi
CML
Incorporation of L-asparagine-14C(UL)
into cellular proteins
Control
Concentration of L-asparagine
0
40
80
120
160
Initial specific activity in incubation medium
(mCi/mM)
DBL 725 5304

Fig. 1.

F. Results

At the extreme right of Table 2 is shown a comparison of normal and chronic myelogenous leukemic leukocytes in regard to rates of cellular uptake, incorporation into proteins, and conversion to $^{14}CO_2$ of L-asparagine-^{14}C(UL). Also shown in Table 2 is the white cell differential pattern. In these experiments cells were incubated with approximately 0.8 μg (1 μCi) of L-asparagine-^{14}C (UL) as the only exogenous source of this amino acid. In the chronic myelogenous cases shown in Table 2 the rates mentioned above were significantly higher than normal. When incorporation into proteins in these cases was expressed as a percentage of total uptake of L-asparagine, the relationship found for the CML's was about 52%, which is less than the average of 62% for the controls.

The cellular uptake and the metabolic rates of leukocytes in the same patient tested on three different occasions while under drug therapy are presented in Table 3.

Figure 1 demonstrates the relationship between incorporation of the tracer into cellular proteins and its initial specific activity (mCi/mM) in the incubation medium for several duplicate samples containing equal radioactivities but different amounts of cold L-asparagine for a leukemic patient and a control.

The results obtained with leukemic and control cells when they are preincubated in a medium containing no additional cold asparagine or 15.8 μg/ml of it are depicted in Table 1. It is obvious that preincubation in a medium containing cold asparagine does not eliminate the differences in the uptake and metabolism of this amino acid by normal and leukemic cells.

Table 2. Cell pattern (differential) and metabolism of L-asparagine-^{14}C (UL) in leukocytes from controls and CML patients

Subject	Diagnosis	Peripheral blood white cell differential														Distribution of radioactivity in units of 10^3 dpm/10^6 cells/ hr/μ Ci of tracer			
		WBC count per cu. mm in thousands	Segmented	Nonsegmented	Metamyelocyte	Myelocyte	Promyelocyte	Eosinophil	Basophil	Lymphocyte	Monocyte	Myeloblast	Megakaryocyte	Eo. myelocyte	Prolymphocyte	Cellular uptake	Incorporation into proteins	$^{14}CO_2$ production	% total cellular uptake incorporated into proteins
1	C	10.1	77	2				2		18	1					14	10	0.7	73
2	C	4.9	63	5				2		24	6					14	9	0.5	64
3	C	7.6	60	2				1		30	7					17	8	1.2	45
4	C	5.4	60	4				6	1	26	3					16	10	1.1	64
5	C	6.4	45	5				3		42	5					18	10	0.6	56
6	C	7.5	68	3					1	25	3					8	5	0.8	67
7	CML	8.6	73	1	2	1		2	5	10	6					27	15	1.4	54
8	CML	17.9	56	3	1	1	1	4	19	9	4	1	1			38	20	1.7	52
9	CML	42.4	49	11	14	15	2	3	1	2	2			1		85	44	4.0	51
10	CML	57.4	39	11	25	11	1	3	3	4	3					95	48	2.8	51

Table 3. Metabolism of L-asparagine-^{14}C (UL) by the leukocytes of patient #9. A serial study for three consecutive weeks.

	Week	1	2	3
	WBC count per cu. mm	42.4	27.0	22.2
White cell differential	Segmented	49	62	59
	Nonsegmented	11	8	3
	Metamyelocyte	14	6	8
	Myelocyte	15	8	7
	Promyelocyte	2	1	2
	Eosinophil	3	2	5
	Basophil	1	8	10
	Lymphocyte	2		2
	Monocyte	2	5	4
	Megakaryocyte			
	Eo. myelocyte	1		
	Prolymphocyte			
Distribution of radioactivity in units of 10^3 dpm/10^8 cells/hr/μCi of tracer	Cellular uptake	85	80	72
	Incorporation into proteins	44	43	39
	$^{14}CO_2$ production	4.0	2.8	2.6
	% total uptake incorporated into proteins	51	53	55

Table 4 shows the incorporation of an essential amino acid L-valine-^{3}H (G) into proteins of chronic myelogenous leukemic leukocytes in the presence or absence of exogenous L-asparagine. No significant differences were observed under these two conditions.

Table 4. Metabolism* of L-valine-^{3}H (G) by leukocytes of CML patients in presence and in absence of L-asparagine in the incubation medium.

Subject	WBC count per cu. mm	Diagnosis	Total cellular uptake		Incorporation into proteins	
			With L-asparagine	Without L-asparagine	With L-asparagine	Without L-asparagine
ELW	20.2	CML	20	20	7	7
SJS	16.1	CML	24	22	10	9
CW	16.6	CML	14	16	5	5

* Units of 10^{3} dpm/10^{8} cells/hr/μCi of tracer.

G. Discussion

In search of an explanation for the significant increase observed in the utilization of exogenous L-asparagine by the leukocytes of CML patients as compared to those obtained from control subjects, the following possibilities can be explored:

a) Do these differences arise from pool-size effects?

b) Are the leukemic cells dependent on exogenous L-asparagine?

c) Do the chronic myelogenous leukemic leukocytes merely use more L-asparagine than the control leukocytes simply due to their generally elevated metabolic activity?

The enhanced rates of uptake and metabolism of leukemic leukocytes cannot be ascribed to pool-size differences between normal and leukemic cells, since preincubation in a medium containing physiological concentrations of L-asparagine does not eliminate the differences in uptake and utilization of exogenous L-asparagine by leukemic and control cells (Table 1).

It is known that the cells which are dependent on exogenous L-asparagine cannot synthesize this amino acid when they are deprived of it. Based on the results of Table 4, the notion of a requirement for L-asparagine in the granulocytic leukemic cells studied may also be ruled out as protein synthesis in L-asparagine-dependent (or L-asparaginase-sensitive) cells is known to be diminished in the absence of extracellular asparagine.[12] However, when L-valine-^{3}H (G) was used as tracer for the leukocytes of CML patients, no significant differences were observed in either uptake or incorporation into cellular proteins in the presence or absence of added L-asparagine

in the incubation medium. This finding is indicative of the independence of the granulocytes of the observed CML patients of L-asparagine and suggests therefore that L-asparaginase should not be expected to be beneficial in the treatment of chronic myelogenous leukemia.

Analysis of the data of Tables 2 and 3 suggests that enhanced uptake of L-asparagine by leukemic cells cannot be accounted for merely by its increased incorporation into proteins and its oxidation. It is likely that more L-asparagine goes also into nucleotides via asparatic acid in CML leukocytes than in controls (see metabolic pathways of L-asparagine included in this section).

The rates of incorporation of other amino acids into the proteins of leukocytes from chronic myelogenous leukemic patients were investigated by others. These rates were found to be higher in chronic myelogenous cases than normals for labelled valine,[19] leucine,[19] glycine,[20] L-cystine,[21] and L-methionine[22] in addition to the L-asparagine of the present assay. These findings suggest an overall enhanced metabolic activity in chronic myelogenous leukemic cells. Sloane et al., who also studied the uptake of L-methionine by leukemic cells, were unable to correlate their results with the peripheral white cell count or the clinical state of the patients. In the present study, however, the rates of uptake, incorporation into proteins, and $^{14}CO_2$ production showed some correspondence with white cell counts of the peripheral blood combined with the clinical state of the patients (Table 2).

In a serial assay with leukocytes from the same patient under drug therapy on three different occasions, the fall in cell counts was accompanied by slow decrease in uptake and metabolic rates, which

remained higher than normal values, thus manifesting the persistence of the leukemic condition of the patient. Inevitably a fundamental question arises as to the reason for the apparently enhanced metabolic activity of the leukemic cells. What are the factors responsible for the increased uptake and utilization of a non-essential amino acid like L-asparagine by leukemic cells? If this increase were only in uptake it would be tempting to search for an answer in possibly altered membrane functions affecting transport processes. The enhanced incorporation into proteins of leukemic cells indicates that not only do they take up more amino acid but also they utilize larger amounts of it. The elevated utilization of L-asparagine may be partially due to the presence of immature cells in the blood of the CML patients. The differences observed in metabolic rates between CML leukocytes and those from controls cannot be exactly correlated to the number of immature cells present. To evaluate the contribution of the immature cells to the observed differences in the metabolic rates it is necessary to obtain a cell population highly enriched in immature granulocytes. At the present time cell separation techniques are not sufficiently refined to do this, so for the time being for comparative metabolic assays we must rely on leukocytes highly enriched in granulocytes but composed of differing granulocytic cell types.

Comparative studies on the metabolism of normal and leukemic cells may yield a means of unravelling some exploitable metabolic differences. If a certain regulatory substance specific to the leukemic cells is responsible for such a disease state, finding a compound which may be essential for the synthesis of this substance will indeed be rewarding.

Deprivation of the leukemic cells of such a compound may provide a means of controlling the disease.

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V. ALTERATION IN OXIDATION OF THE METHYL CARBON ATOM OF L-METHIONINE BY LEUKOCYTES IN SCHIZOPHRENIA

A. Summary

Genetic etiology of schizophrenia has been described by several researchers.[3,35,36,37] Moreover, abnormal methylation processes have been implicated in mental disorder of this type, suggesting a metabolic basis for the disease. The present experiments were designed to test possible alteration of leukocyte metabolism in vitro, of the methyl carbon atom of L-methionine-methyl-^{14}C (the major methyl donor in metabolic reactions) in schizophrenia. The relationship was observed between $^{14}CO_2$ produced by leukocytes, from individuals with no medical history of mental disorder and from psychiatric patients, and the concentration of L-methionine in the incubation medium. Differences in amounts of oxidation suggest altered metabolism of the methyl carbon atom of L-methionine in schizophrenic patients investigated in this assay. Similar differences could not be established in case of oxidation of the carboxyl carbon atom of L-methionine-carboxyl-^{14}C.

A viability test was applied to the cells subjected to different doses of chlorpromazine. At non-toxic doses this drug was found to produce varying effects on the oxidation of the methyl carbon atom of L-methionine. However, chlorpromazine did not produce any observable change in the oxidation of the carboxyl carbon atom of the same amino acid.

A considerable overlap was observed between incorporation of label from L-methionine-methyl-^{14}C into proteins of leukocytes from control subjects and schizophrenics.

Incorporation of the label from L-methionine-carboxyl-^{14}C into

cellular proteins was in both cases similar but less than that from the methyl carbon atom, suggesting transfer of the label of the methyl group to some other amino acids which could be incorporated into proteins.

The findings described presently provide additional evidence of involvement of disturbances of methylation in schizophrenia.

B. Introduction

Although schizophrenia has been known for nearly a century, its etiology still remains unknown. At present only symptomatic treatment can be offered to the patient without direct interference with the underlying cause of this illness. Knowledge of its etiology may not only lead to agreement among psychiatrists as to its meaning and its diagnosis, but more significantly may also make possible a radical treatment which can alter the life style of so many people affected by it. Various origins, metabolic, psychogenic, or immunological have been cited for it by different observers.

In the closing paragraph of the preface of his classic treatise in 1884, Thudichum, the founder of modern neurochemistry, describes "many forms of insanity" as "the external manifestation of the effects upon the brain-substance of poisons fermented within the body."[1]

As early as 1896 schizophrenia was included among metabolic disturbances by Kraeplin. Bleuler's hypothesis (1911)[2] favoured this view, and much later Karlsson[3] strongly supported the hereditary nature of this disease. Bleuler's hypothesis that schizophrenia is a metabolic disorder with psychological manifestations eventually triggered research oriented toward study of biochemical changes in schizophrenics. Gradually, it became more widely accepted that the chemical action of certain endogenous organic decomposition products could be held responsible for the manifest mental state in schizophrenia. Opponents of this view insisted on psychogenic origin of the disease.[4] Many believed in other toxic theories. For example, Berger discovered a substance in the blood of catatonics that had stimulatory effect on

the motor centres of dogs. Several other toxic theories were propounded only to be subsequently refuted. At one stage, production of schizophrenic toxin was attributed to the thyroid gland. The sexual disturbances it causes led some observers to implicate the function of reproductive organs. Yet others resorted to infection, such as tuberculosis, in explaining psychosis. Bruce, Dide, and Sacquepée[5] claimed to have found bacteria in the bloodstream of schizophrenics and also noted leukocytosis in catatonia. Dide frequently found insufficient detoxification due to fatty degeneration of the liver.

In 1933 Quastel and Wheatley[6] investigated the effect of mescaline and other aromatic amines on the oxidation of glucose, lactate, pyruvate, glutamate, and succinate by brain tissue in vitro. Inhibition was observed in all except succinate. The finding that mescaline caused inhibition of oxidation in a way similar to narcotics, which could be abolished by succinate, led them to suspect inadequate detoxification of similar substances by the liver as the cause of their recirculation in the bloodstream, giving rise to reactions with psychological manifestations.

Osmond and Smythies' work in 1952 showed a remarkable similarity between schizophrenia and symptoms of toxicity of mescaline, an alkaloid from the peyote plant which was first isolated from mescal buttons by Heffter in 1896. Could then mescaline be considered a likely agent for psychotic manifestations observed in schizophrenia?[7]

In the early 1950's the similarity in chemical structure of epinephrine and mescaline was noted.[8] It was postulated that in the biogenesis of epinephrine by transmethylation of norepinephrine by the $-CH_3$ group from major methyl donors methionine or choline, disorder in transmethylation

might lead to O-methylated compounds. It is noteworthy to mention here that 18 years earlier Noteboom had pointed out that 3, 4-dimethoxyethylamine was found to be most potent among the phenylethylamine derivatives tested in producing catatonia in experimental animals.[9] Since high dosages are required to produce catatonic effect, it was doubted that large enough amounts of mescaline could be accumulated in the body, as a result of defective transmethylation, to cause the mental picture seen in schizophrenics.

Osmond's hypothesis of an endogenous compound, possibly resulting from an abnormality of epinephrine metabolism and Harley-Mason's prediction of 3, 4-dimethoxyphenylethylamine as a cause of schizophrenic psychosis inspired some with attempts to isolate such a substance from the body fluids of acute schizophrenics. The action of some biologically active amines related to epinephrine had already been studied in experimental animals.[10] One of the compounds, namely dimethoxyphenylethylamine (DMPEA), unlike others had been found not to elicit sympathomimetic response. Although p-methoxy-phenylethylamine, m-methoxy-phenylethylamine, and methylene-dioxy-phenylethylamine stimulate CNS, in contrast, DMPEA depresses it. Later in 1959 it was demonstrated that 3, 4-dihydroxyphenylethylamine can be metabolized by the action of 3, 0-methyltransferase in brain homogenates.[11] In the same year 3, 4-dihydroxyphenylethylamine was shown to undergo 4, 0-methylation in liver homogenates <u>in vitro</u> in the presence of S-adenosylmethionine.[12] Moreover, in 1962 a compound was isolated from the urine of schizophrenic patients, and was identified as DMPEA.[13] This led to the stronger implication of excessive methylation as a

biochemical cause for schizophrenia. If DMPEA represented the abnormal metabolite, this would suggest the presence of the enzyme 4,0-methyltransferase in schizophrenics.

In 1967 preferential 4-demethylation of DMPEA was observed after injection of 3-$^{14}CH_3$ and 4-$^{14}CH_3$ DMPEA separately to rats.[14] Based on the apparent importance of the 4-methyl component in known psychotogens, possible presence of an enzymatic deficiency leading to a defect in 4-demethylation of DMPEA in schizophrenics was suggested.

Intensified research to show the presence of DMPEA in the body fluids yielded the following conflicting conclusions:

(1) There is DMPEA in the urine of schizophrenics only.[15,16]

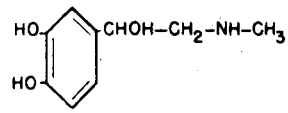
(2) There is no DMPEA in human urine.[17]

(3) DMPEA, popularly known as the "pink spot," exists in the urine of both schizophrenics and normals without significant differences between them.[18]

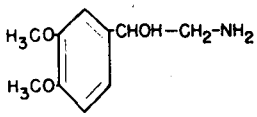
The effect of diet and phenothiazine drug intake on urinary DMPEA[19] and differences in techniques of isolation and detection may be factors contributing to the nonuniformity of these conclusions. One thing is now clear: that DMPEA, when administered even in large doses, does not evoke any psychological changes in man.[20,21]

Although DMPEA, which may exist in small quantities both in normal and schizophrenic urine, is no longer considered to be the chemical psychotogen, the notion that disturbances in methylation may lead to psychological aberrations still prevails. Some methylated compounds implicated in psychosis are shown in Fig. 1. The following are some reasons for implicating disturbances in transmethylation

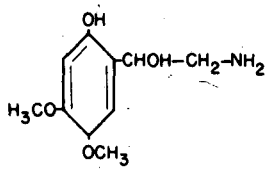
Chemical structure of Epinephrine and some methylated compounds implicated in schizophrenia



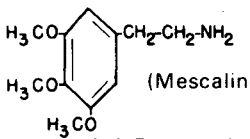
Epinephrine



3,4-dimethoxyphenylethanolamine

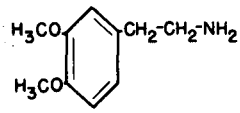


2-hydroxy-4,5-dimethoxyphenylethanolamine

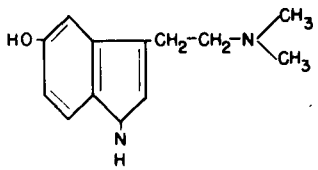


(Mescaline)

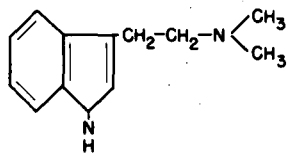
3,4,5-trimethoxyphenylethylamine



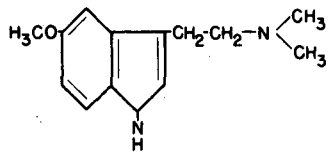
3,4-dimethoxyphenylethylamine (DMPEA)



Bufotenin



N-dimethyltryptamine



5-methoxy-N-dimethyltryptamine

DBL 725 5303

Fig. 1.

in schizophrenia:

(1) Mescaline, which produces psychotoxic manifestations similar to schizophrenia, is a methylated compound.[7]

(2) Psychotoxic compounds, whether indolic or not, are found to be methylated[22] and N- and O- methylations are important metabolic processes.[23]

(3) Exacerbation of mental and behavioral symptoms in schizophrenics after administration of loading doses of cysteine and MAO (monoamineoxidase) inhibitors are found to coincide with the accumulation in the urine of the tryptamine methylation product N-dimethyltryptamine (DMT) and serotonin methylation products, bufotenin, and 5-methoxy-N-dimethyltryptamine (5-MeODMT), all three of which are considered to be psychotoxic.[24,25]

(4) 3, 4-dimethoxyphenylethanolamine and 2-hydroxy-4, 5-dimethoxyphenylethanolamine have been proposed to be two possible psychotogens[26] as a result of β-hydroxylation and methylation of 6-hydroxydopamine. 6-hydroxydopamine, on the other hand, has been described as the aberrant metabolite with some role in causing schizophrenia.[27]

(5) Methionine, a metabolically active donor of methyl groups, is a methylated compound that causes aggravation of schizophrenia, and manifests psychotoxicity in normals when administered in high doses.[28,29,30]

(6) High doses of nicotinamide or nicotinic acid, methyl acceptors, have been found to produce therapeautic effects in schizophrenia.[31]

It is argued that by accepting the methyl groups these vitamins make them unavailable for the formation of methylated psychotogens.

(7) Exacerbation of conditions of schizophrenic patients treated with simultaneous administration of L-methionine or L-cysteine with a MAO inhibitor has been observed.[32,33] MAO inhibitor is expected to prevent the metabolic destruction of some methylated compounds.

Because methylation has been implicated for such a long time in schizophrenia, a comparative study of oxidation of the methyl carbon of the major methyl donor methionine was recently undertaken in normal, schizophrenic, and depressive subjects.[34] L-methionine-methyl-^{14}C was administered intravenously, and the breath was analysed for $^{14}CO_2$. Differences in the rates of oxidation of methyl carbon of methionine in groups of people falling into various categories were reported.

The present work searches for some additional evidence concerning the importance of methylation and demethylation in schizophrenic and affective disorders at the cellular level in vitro. Human leukocytes, because they possess mitochandria and are readily available, were chosen as a biopsy specimen for investigation of alteration in oxidation of the methyl carbon of L-methionine-methyl-^{14}C in depressive and schizophrenic individuals when compared with controls.

C. Materials and Methods

Leukocytes were separated from fresh blood drawn from fasting psychiatric patients and from control individuals with no manifest psychosis. The patients were examined and diagnosed by Dr. Tod H. Mikuriya.

The method of isolation of leukocytes from whole blood is included in Section II. At the end of separation the cells were suspended in a special medium, MEM (Gibco), free from L-methionine. Each sample contained $(1.5\text{-}2.5)10^7$ leukocytes in 3 ml MEM contained in a 25 ml Erlenmeyer flask fitted with a rubber top. The samples were preincubated for 30 min. Varying amounts of L-methionine-methyl-^{14}C (specific activity 11 mCi/mM, obtained from New England Nuclear Corp.) were administered into various flasks. The cells, suspended in the medium with varying concentrations of L-methionine, were incubated for 2 hours at 37°C. At the end of the incubation period metabolic reaction was stopped by the addition of 2 ml of acetate buffer at pH = 3.7, $^{14}CO_2$ was collected, and the radioactivity of $^{14}CO_2$ evolved by the cells was measured as previously described in Section II. Counts were converted into dpm's which were normalized to 10^8 cells; dpm/10^8 cells/hr was plotted against amount of L-methionine in the incubation medium.

Following $^{14}CO_2$ collection, each sample was analysed for incorporation of the label from L-methionine-methyl-^{14}C into cellular proteins. The cell debris was centrifuged and washed twice. The TCA precipitate was assayed for its radioactivity as described in Section II.

The procedure described above was repeated for experiments with L-methionine-carboxyl-^{14}C (specific activity 58 mCi/mM, obtained from Amersham/Searle). The specific activity of L-methionine-1-^{14}C

was adjusted to 11 mCi/mM by the addition of cold L-methionine.

The effect of chlorpromazine (Thorazine ®, Smith Kline and French Labs., Philadelphia) on the oxidation of methyl and carboxyl carbon atoms of L-methionine was investigated. The dose effect of this tranquilizer on cell viability was initially determined. Varying amounts of chlorpromazine were added to a flask containing approximately 2×10^7 cells suspended in 3 ml MEM. Viability of the cells exposed to chlorpromazine was assessed by the trypan blue exclusion test already mentioned in Section II. Chlorpromazine was administered to the cells in amounts well below the toxic level and its effect on oxidation was investigated.

D. Results

When the concentration of L-methionine added to the incubation medium was varied, $^{14}CO_2$ produced as a result of metabolism of L-methionine-methyl-^{14}C by the leukocytes of the schizophrenic patients was found to differ from that for the control subjects (Fig. 2). In all except two cases $^{14}CO_2$ production by the leukocytes of the schizophrenics was lower than by those from the controls. Table 1 summarizes the history and drug therapy of the patients who participated in the present assay.

The amount of oxidation of the methyl carbon atom of L-methionine-methyl-^{14}C went up with the increase in the incubation time. This result is shown in Fig. 3 for a control subject and a patient, indicating retention of the initial viability of the cells. Unlike the metabolic difference with the methyl-labelled methionine, no difference in $^{14}CO_2$ production from the carboxyl carbon atom of L-methionine-carboxyl-^{14}C

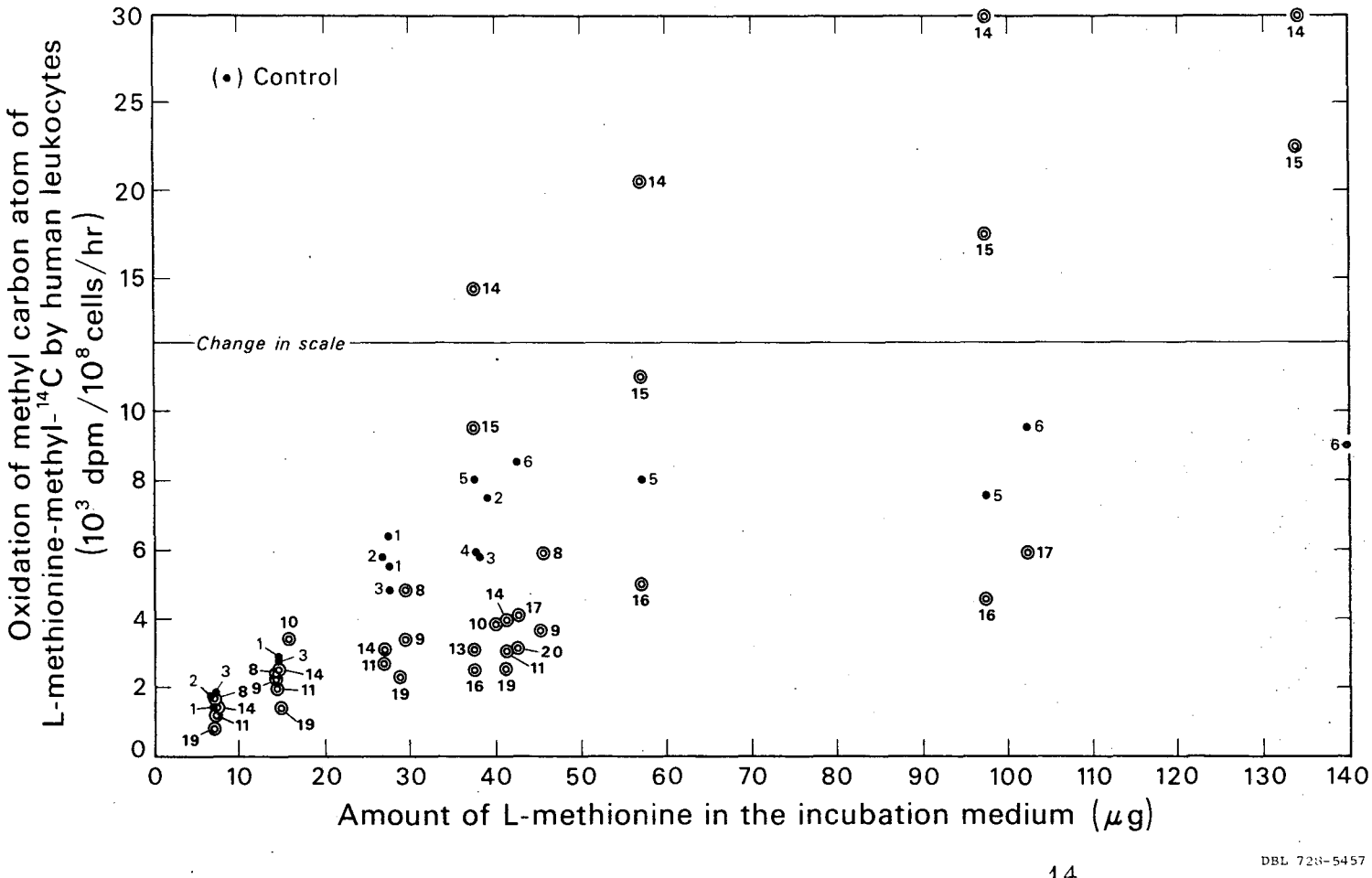


Fig. 2. Oxidation of the methyl carbon atom of L-methionine-methyl-^{14}C by the leukocytes of control subjects and psychiatric patients at different methionine concentration in the incubation medium. For diagnosis of the patients refer to Table 1.

Table 1. Summary of patients' history.

Patients' number	DIAGNOSIS	Age	Sex	Auditory Hallucination	Disturbance of affect	Thought disorder	Depression	Duration of illness (years)	DRUG THERAPY Drug		
8	Mental retardation Chronic undifferentiated	25	F	+	0	+	0	4	Haloperidol	1 mg	1BID
9	Schizo-affective	38	F	?	+	+	+	16	Fluphenazine	2.5 mg	1TID
									Methyprylon	300 mg	1HS PRN
10	Chronic undifferentiated	40	M	?	+	+	+	7	Mesoridazine	50 mg	1BID
11	Mental retardation	19	M	+	+	+	+	10	Fluphenazine	2.5 mg	1BID
12	Schizo-affective, depressed	51	F	+	+	+	+	> 3	Amitriptyline-perphenazine	2-25	1TID
									Pentobarbital	100 mg	1HS
									Trihexyphenidyl hydrochloride	2 mg	1TID
									Diphenhydramine	50 mg	1Q^{4h} PRN
13	Residual schizophrenia	36	M	+	+	+	+	> 14	Niacinamide	0.5 mg	1BID
14	Chronic undifferentiated	21	F	+	0	+	0	5	Thioridazine	100 mg	1QID
									Ascorbic acid	100 mg	1QID
15	Chronic undifferentiated	42	F	?	0	+	+	6	Thioridazine	50 mg	1HS
									Protriptyline hydrochloride	10 mg	1BID
									Chloral hydrate	0.5 mg	1HS PRN
16	Chronic undifferentiated	26	F	+	+	+	+	5	None		
17	Residual schizophrenia	33	M	0	0	+	+	15	None		
18	Schizo affective, circular	26	M	0	+	+	+	20	Trifluoperazine hydrochloride	2 mg	1BID
									Diazepam	5 mg	1Q^{4h} PRN
19	Residual schizophrenia	47	F	0	0	0	0	> 10	None		
20	Chronic undifferentiated	22	F	+	+	+	0	3	Haloperidol	2 mg	1BID
									Diazepam	5 mg	1TID

+: Yes
0: None or not significant
?: Unknown

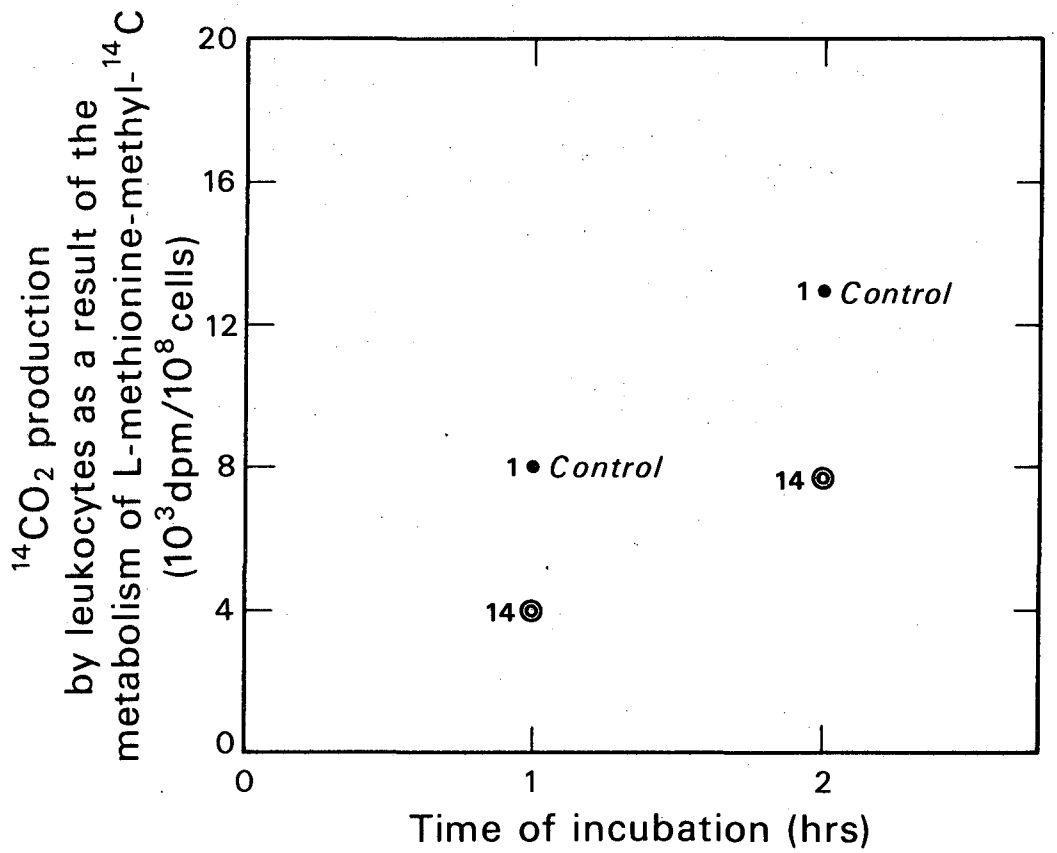


Fig. 3. $^{14}CO_2$ production from oxidation of the methyl carbon atom of L-methionine-methyl-^{14}C during different incubation periods. Counts were converted into dpm and normalized for 10^8 cells.

by the leukocytes of the psychiatric patients and the controls was observed (Table 2).

Incorporation of the label from methyl ^{14}C-labelled methionine into cellular proteins exhibited considerable overlap in the patients and the controls (Fig. 4). Furthermore, incorporation of label from L-methionine-carboxyl-^{14}C into proteins was found to be less than that from L-methionine-methyl-^{14}C (Fig. 5).

The weighted least-squares best fits of a function of the form $ax/b+x$ to the measurements of 1) $^{14}CO_2$ production 2) incorporation of the label into proteins by the leukocytes of 11 of the 13 psychiatric patients (excluding patients #14 and #15, see discussion) and 7 controls as a result of the metabolism of L-methionine-methyl-^{14}C are shown in Figs. 6 and 7 respectively.

The effect of chlorpromazine on the viability of leukocytes was found to be variable. In two patients tested, a sharp loss of viability of the cells was noted at a concentration of 10 μg chlorpromazine in 3 ml incubation medium. In a control, on the other hand, viability was retained by the cells at much higher doses of this tranquillizer administered to the cell suspension in vitro, as shown in Fig 8.

Non-toxic levels of chlorpromazine produced varied effects on the oxidation of the methyl carbon atom of L-methionine but remained ineffective in the case of oxidation of its carboxyl carbon atom (Table 3). For the cells from patient #13, doses of 0.25 μg or 0.5 μg chlorpromazine in the incubation medium resulted in an increase in $^{14}CO_2$ production. In another patient (#17) chlorpromazine did not appreciably change the oxidation of methyl carbon atom although it produced significant decrease

Table 2. Oxidation* of the carboxyl carbon atom of L-methionine-carboxyl-^{14}C by leukocytes from controls and schizophrenic patients.

Subject	Diagnosis	Oxidation to $^{14}CO_2$
4	Control	3.0
5	Control	2.9
15	Chronic undifferentiated	3.8
20	Chronic undifferentiated	2.9

* Units of 10^3 dpm/10^8 cells/hr/3μCi tracer.

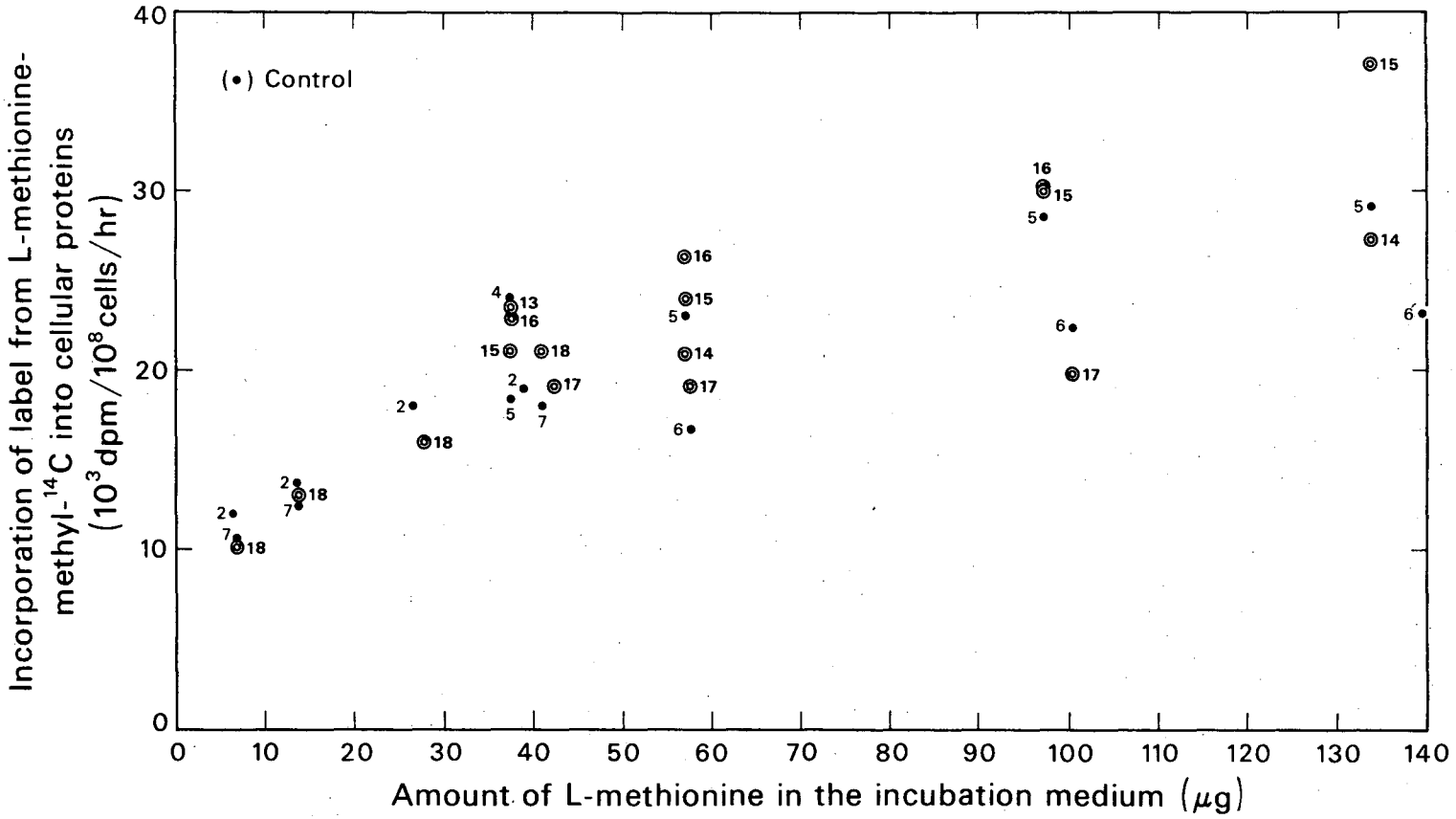


Fig. 4. Incorporation of the label from L-methionine-methyl-^{14}C by leukocytes into cellular proteins as a function of L-methionine in the incubation medium. For diagnosis of the patients Table 1 should be consulted.

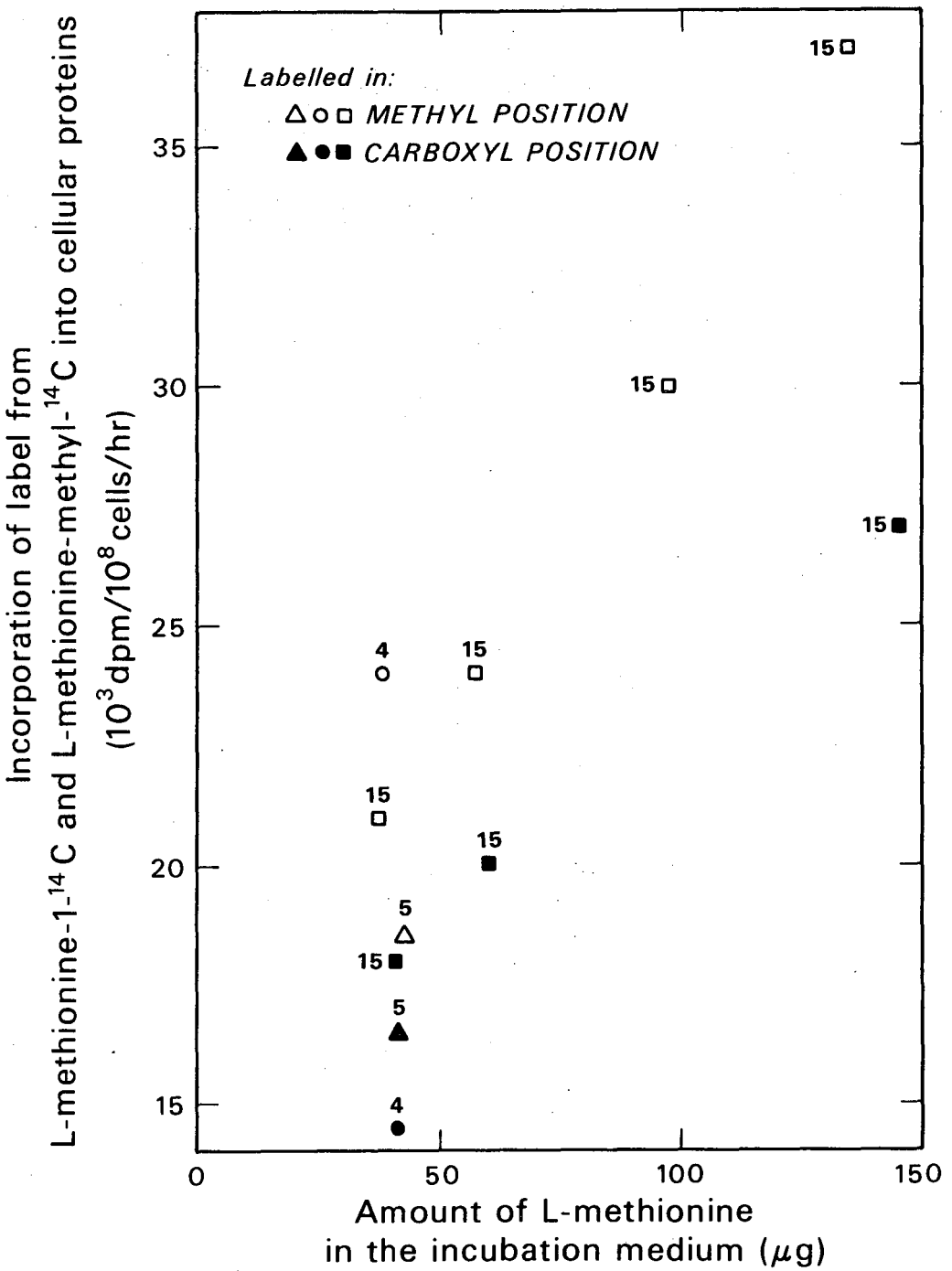


Fig. 5. Comparison of incorporation of the label from L-methionine-1-^{14}C with that from L-methionine-methyl-^{14}C into cellular proteins at different L-methionine concentrations of the incubation medium.

Fig. 6. The weighted least-squares best fit of a function of the form $ax/b+x$ to the measurements of $^{14}CO_2$ production by the leukocytes of psychiatric patients (A) and controls (B) as a result of the oxidation of the labelled atom of L-methionine-methyl-^{14}C. x represents the amount of L-methionine in the incubation medium. The fitted values of a and b are as follows:

a = 6500 and b = 32.6 for the patients,

a = 12 000 and b = 35.5 for the controls.

From this plot a great difference between the two groups is evident.

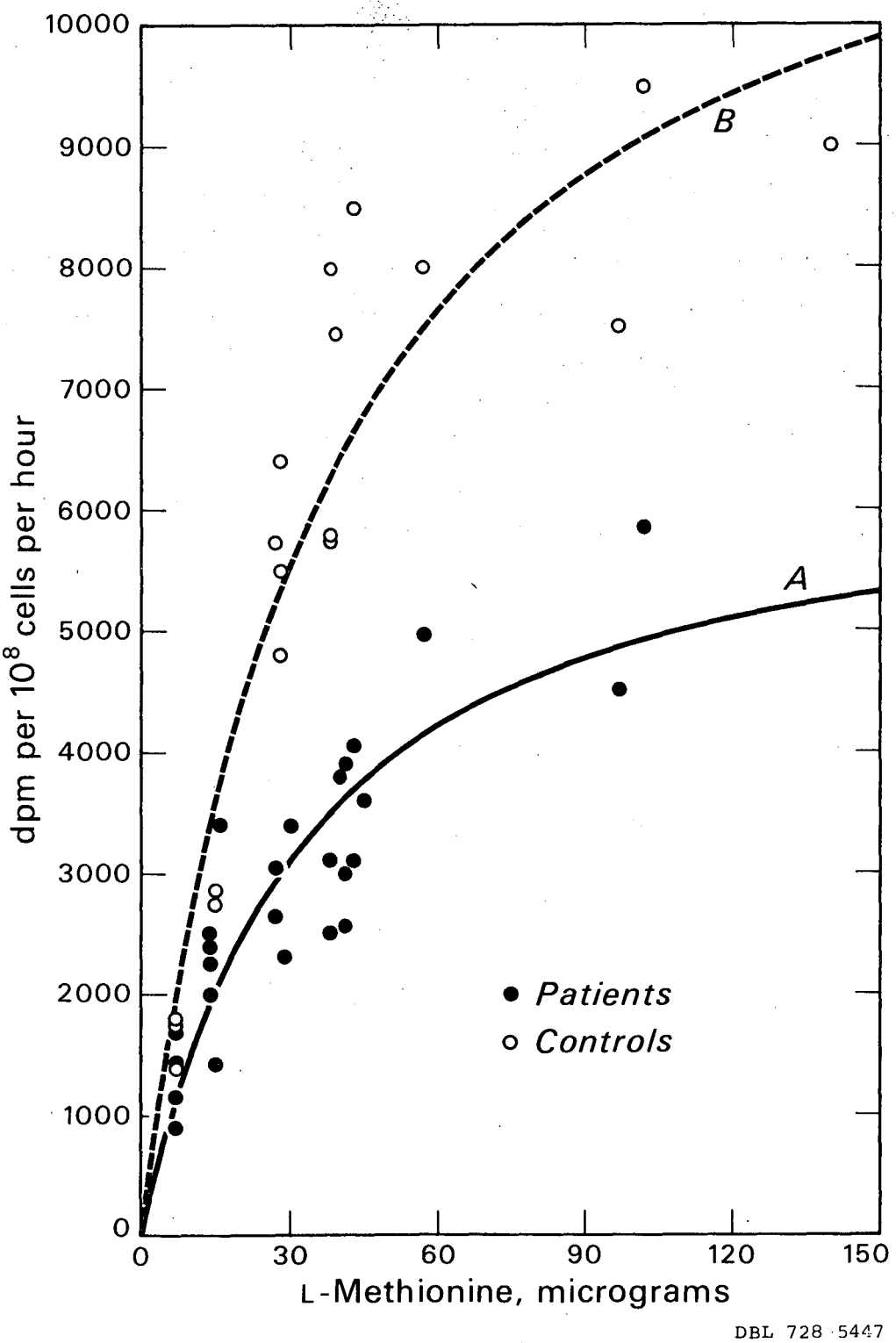

10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
dpm per 10^8 cells per hour
0
30
60
90
120
150
L-Methionine, micrograms
B
A
Patients
Controls
DBL 728 5447

Fig. 6.

Fig. 7. The weighted least-squares best fit of a function of the form $ax/b+x$ to the measurements of the incorporation of the label from L-methionine-methyl-^{14}C into proteins by the leukocytes of psychiatric patients (A) and controls (B). x represents the amount of L-methionine in the incubation medium. The fitted values of a and b are as follows:

$a = 30\,000$ and $b = 18.5$ for the patients,

$a = 28\,000$ and $b = 15.3$ for the controls.

The closeness of the two curves is obvious.

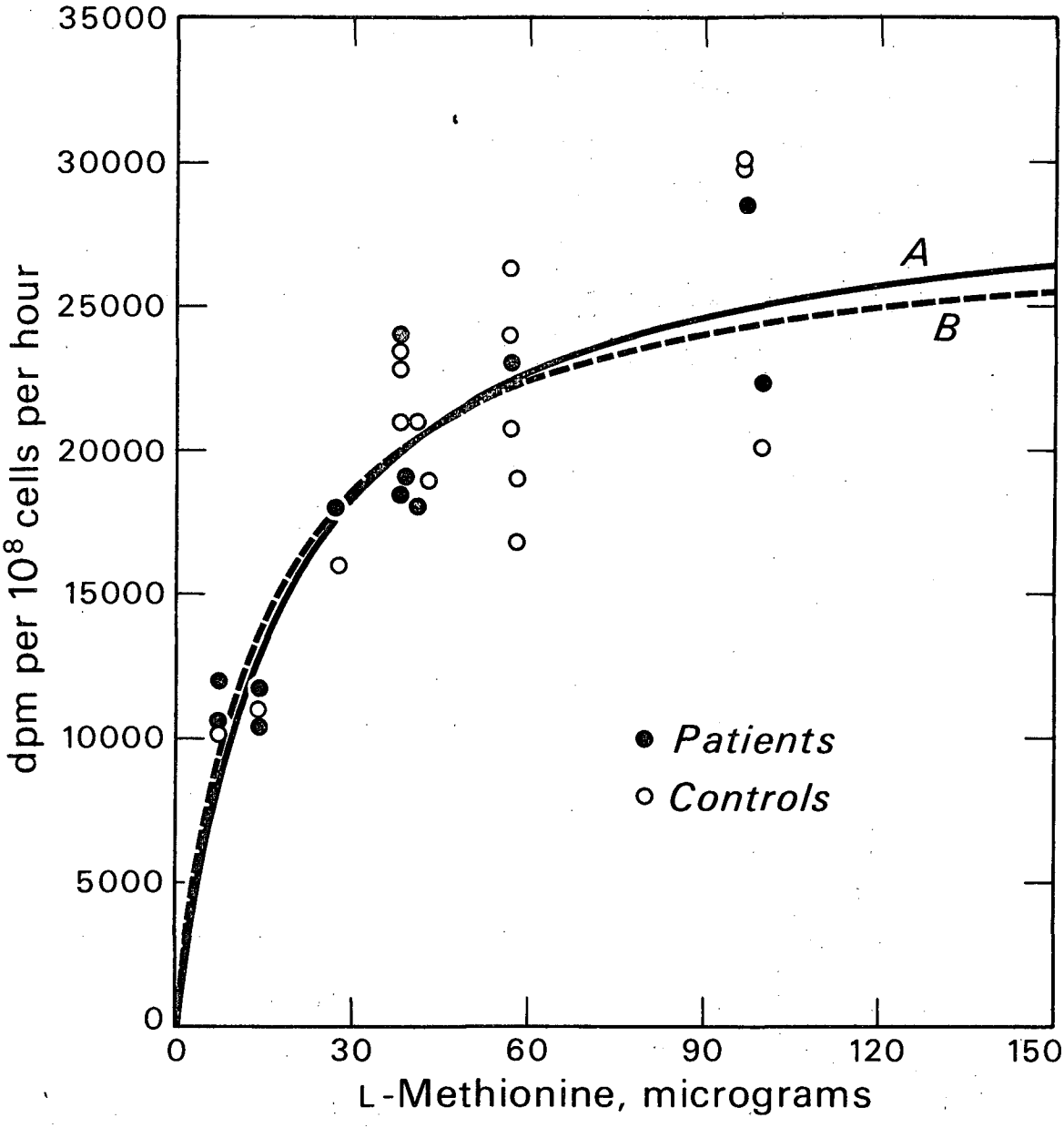

35000
30000
25000
20000
15000
10000
5000
0
dpm per 10^8 cells per hour
0
30
60
90
120
150
L-Methionine, micrograms
A
B
Patients
Controls
DBL 728-5448

Fig. 7.

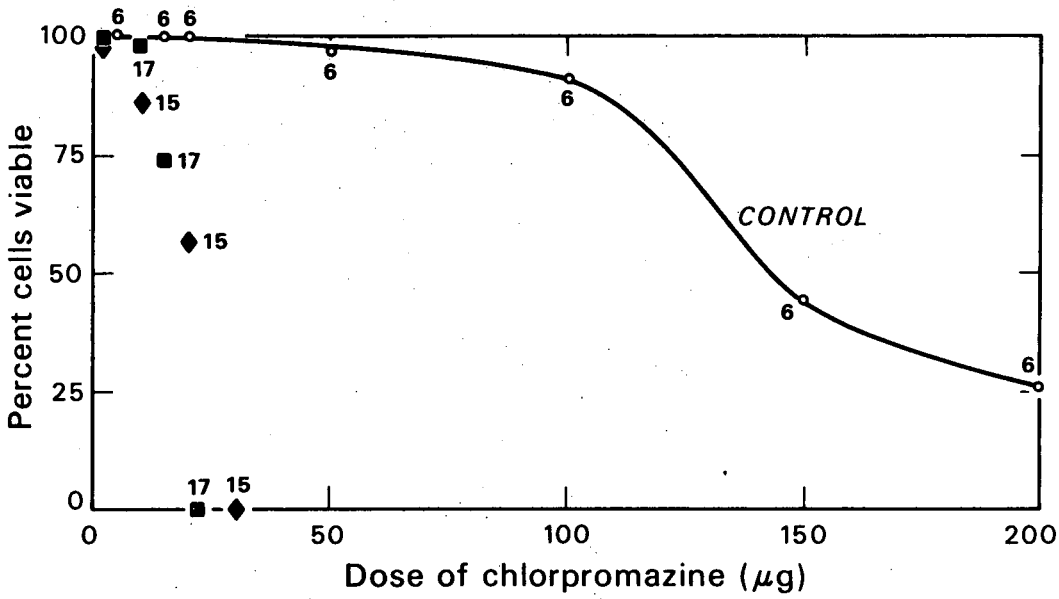


Fig. 8. Effect of chlorpromazine concentration on the viability of the leukocytes isolated from blood of a control subject and two psychiatric patients. Each sample contained approximately 2×10^7 cells in 3 ml MEM and varying doses of chlorpromazine. Viability was assessed by dye exclusion test.

Table 3. Effect of chlorpromazine (Thorazine) on L-methionine-methyl-^{14}C metabolism* by leukocytes in vitro.

Subject #	Dose of Thorazine (μg)	Diagnosis	Oxidation to $^{14}CO_2$ With Thorazine	Oxidation to $^{14}CO_2$ Without Thorazine	Incorporation of label into proteins With Thorazine	Incorporation of label into proteins Without Thorazine
13	0.25	Residual schizophrenic	8	3	14	23
13	0.5		10	3	13	23
4	0.13	Control			23	24
5	2	Control			19	19
6	2	Control			11	12
17	2	Residual schizophrenic	5	4	11	19
20	0.1	Chronic undifferentiated schizophrenic	5	3		

* Units of 10^3 dpm/10^8 cells/hr.

in protein incorporation of the label. In the leukocytes of the control subjects, administration of 0.13 or 2 μg of chlorpromazine to approximately 2×10^7 cells in 3 ml MEM did not produce any significant change in protein incorporation of the label from L-methionine-methyl-^{14}C. In the cells of all patients tested for this purpose significant decrease in protein incorporation was observed in the presence of chlorpromazine.

E. Discussion

Any attempt to evaluate the results of this section necessitates a careful consideration of the metabolic pathways of L-methionine. The major metabolic functions of this amino acid shown in Fig. 9, are as follows:[38] 1) incorporation into cellular proteins, 2) formation of S-adenosylmethionine which is an active methyl group donor, and 3) conversion into cystathionine, cysteine, and other derivatives. Methionine is converted into S-adenosylmethionine which is in turn transformed into S-adenosylhomocysteine by the removal of the methyl group of methionine. S-adenosylhomocysteine is then converted into homocysteine which can either be methylated to give rise to methionine or be further transformed irreversibly into cystathionine which is eventually cleaved to α-ketobutyric acid and cysteine. Oxidative decarboxylation of α-ketobutyric acid results in the formation of propionyl-CoA which is eventually metabolized to succinyl-CoA, an intermediate of the tricarboxylic acid cycle. CO_2 evolved as a result of the decarboxylation of the α-ketobutyric acid, originating from the metabolism of L-methionine, is derived from the carboxyl group of L-methionine. $^{14}CO_2$ produced from the metabolism of L-methionine-carboxyl-^{14}C is represented in Fig. 10.

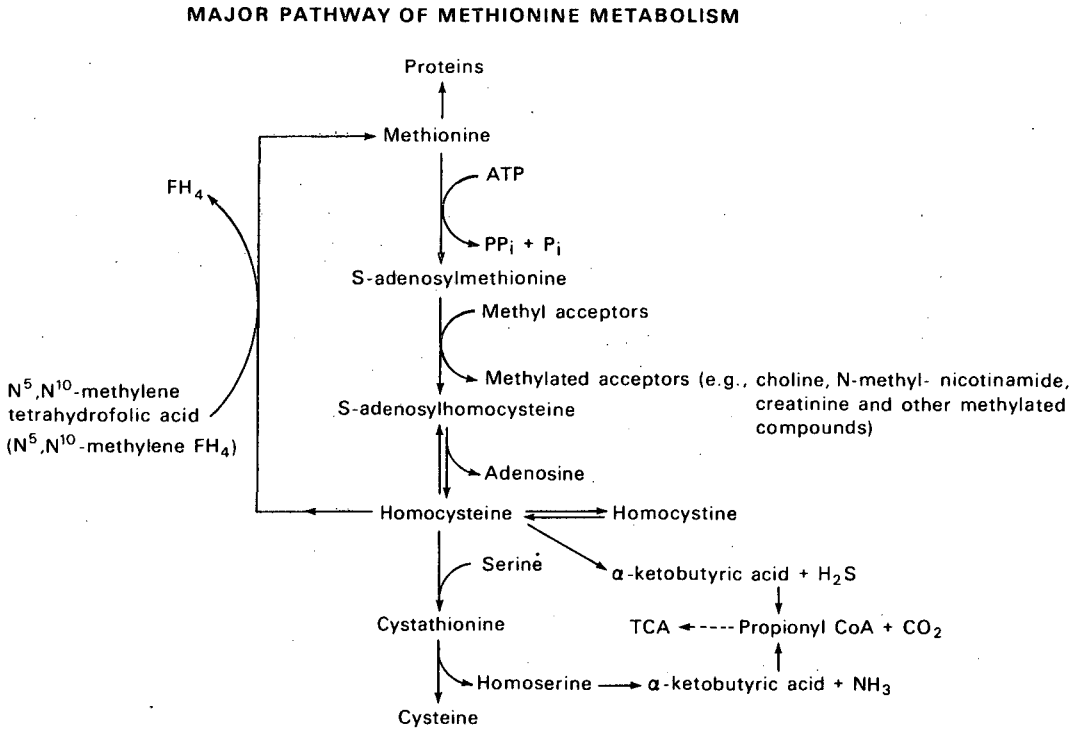

MAJOR PATHWAY OF METHIONINE METABOLISM
Proteins
Methionine
ATP
PPi + Pi
S-adenosylmethionine
Methyl acceptors
Methylated acceptors (e.g., choline, N-methyl- nicotinamide, creatinine and other methylated compounds)
S-adenosylhomocysteine
Adenosine
FH4
N5,N10-methylene tetrahydrofolic acid
(N5,N10-methylene FH4)
Homocysteine
Homocystine
Serine
α-ketobutyric acid + H2S
Cystathionine
TCA
Propionyl CoA + CO2
Homoserine
α-ketobutyric acid + NH3
Cysteine

Fig. 9.

METABOLIC PATHWAY LEADING TO THE OXIDATION OF THE CARBOXYL CARBON ATOM OF METHIONINE

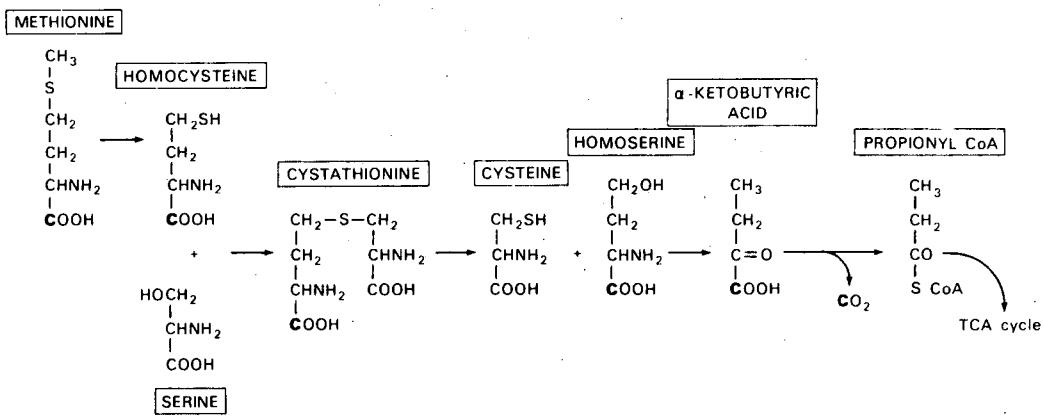


Fig. 10.

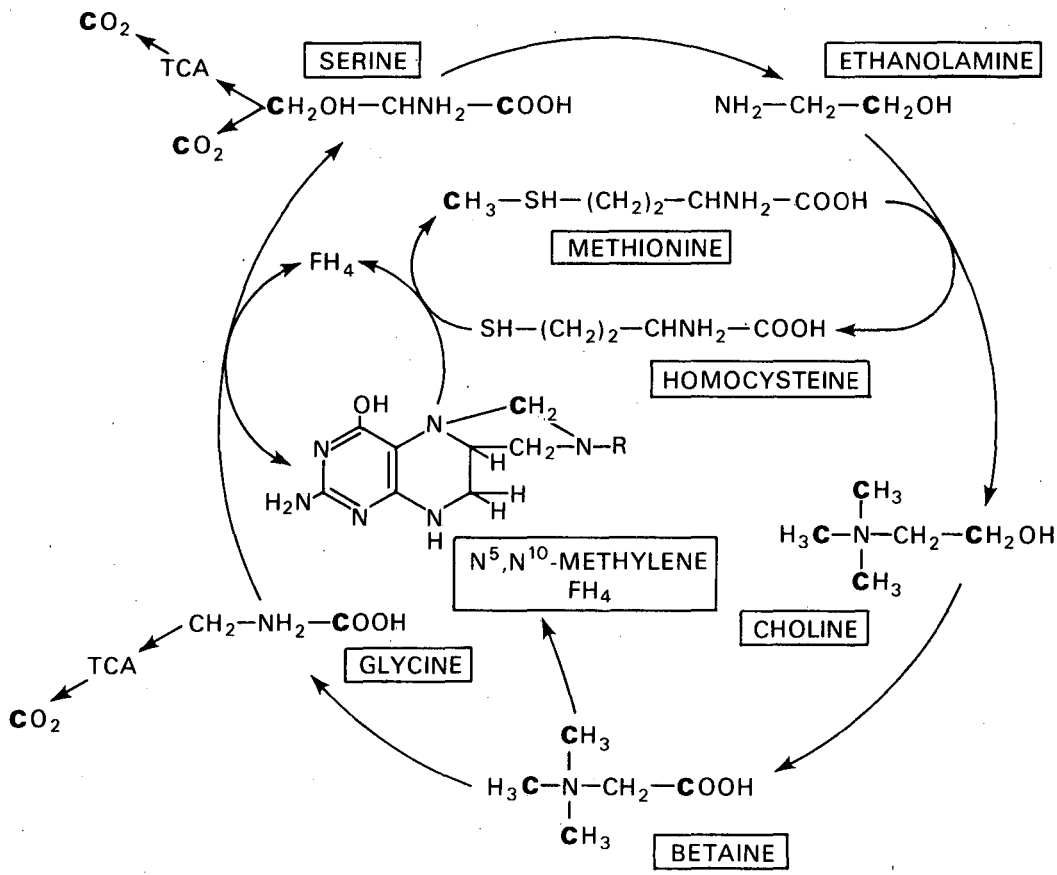
The metabolic fate of the methyl carbon atom of L-methionine-methyl-^{14}C is outlined in Fig. 11. The label of this methyl carbon atom can appear in 1) methylated compounds, 2) $^{14}CO_2$, and 3) proteins through incorporation of L-methionine-methyl-^{14}C and other amino acids subsequently labelled by ^{14}C from the methyl group of L-methionine.

The curves representing $^{14}CO_2$ production from the methyl carbon atom of L-methionine-methyl-^{14}C by the leukocytes of the psychiatric patients as a function of L-methionine concentration in the incubation medium appear to fall into two groups (Fig. 2). The members of one group lie below the control curves and those for the other above it. In all cases except one (patient #14) CO_2 production tends to plateau at high concentration, most probably due to saturation phenomena.

If indeed, some forms of schizophrenia can be related to abnormal methylation products, as greater amounts of methyl groups are diverted into production of these methylated compounds, $^{14}CO_2$ production would then be diminished. The curves lower than those for controls (Fig. 2) comply with such an expectation. Two of the $^{14}CO_2$ curves for the schizophrenic patients (Fig. 2), however, reveal values much above the control levels. It is striking that these two patients with CO_2 curves higher than those for controls were both taking thioridazine (Mellaril ®). Moreover, patient #14 with the highest $^{14}CO_2$ curve was receiving a higher dose of this drug than patient #15 (Table 1). Therefore the possibility of a drug effect cannot be excluded.

In some cases exacerbation of the psychotic condition in schizophrenia has been reported when patients are given large oral doses of

THE PATHWAY SHOWING THE MAJOR SITES OF CO_2 PRODUCTION FROM ^{14}C LABELED METHYL CARBON ATOM OF L-METHIONINE



C = LABELED CARBON ATOM
TCA = TRICARBOXYLIC ACID CYCLE
FH_4 = TETRAHYDROFOLIC ACID

Fig. 11.

L-methionine in the presence of a monoamine oxidase inhibitor.[29,33] These so-called "methionine reactors" may initially belong to the group with lower ability to oxidize the methyl carbon atom of L-methionine.

At this juncture it must be pointed out that all the patients except three (#16, #17, #19) were under drug therapy at the time of these experiments. The mechanisms by which these drugs produce their effects are not at present fully understood. It is noteworthy, however, that for those three patients who were not under treatment, $^{14}CO_2$ curves obtained were lower than those for the controls. This was especially true for patient #19, with a past psychiatric history but not under medication for a long time prior to the experiment, who had the lowest $^{14}CO_2$ curve. Evidently no tranquillizer could be held responsible in these untreated cases for the depressed $^{14}CO_2$ curves (Fig. 2). In patient #13, diagnosed as residual schizophrenic, it is tempting to attribute the fairly low $^{14}CO_2$ production to huge doses of nicotinamide taken by the patient. It is conceivable that a greater number of CH_3 groups is taken up by nicotinamide to form N-methyl-nicotinamide thus making fewer methyl groups available for oxidation.

Values of $^{14}CO_2$ produced from oxidation of the carboxyl carbon atom of L-methionine were significantly lower than those from the methyl carbon atom both in the schizophrenic patients and the controls. Moreover, the patients could not be separated from the controls according to the amounts of oxidation of the carboxyl atom of L-methionine-carboxyl-^{14}C (Table 2). This finding suggests that the pathway leading to the oxidation of the carboxyl carbon atom of L-methionine may not be affected in schizophrenia.

The results presented here lend support to a hypothesis of disturbances of methylation in schizophrenia. However, for these studies to be more conclusive, more untreated patients free from drug effects and adequately controlled for diet have to be investigated. A systematic study of the initially untreated patients who could be followed up after initiation of drug therapy may provide an elegant means of getting information about the extent of involvement of methylation processes in schizophrenia. Furthermore, the effect of various types of drugs on oxidation of the methyl carbon atom of L-methionine could be studied. To evaluate genetic effects, close relatives of probands should also be systematically tested.

It is evident from Figs. 4 and 7 that the patients and controls cannot be separated according to the curves obtained for incorporation of the label from L-methionine-methyl-^{14}C into proteins.

It is interesting to note that patient #14 with the highest $^{14}CO_2$ curve (Fig. 2) has not an unusually high or low protein incorporation of the label from L-methionine-methyl-^{14}C by the leukocytes. These results suggest that the pathway leading to the incorporation into proteins of the label from the methyl carbon atom of L-methionine may not be altered in schizophrenics. Also, this proves that the difference in appearance of $^{14}CO_2$ from methyl labelled methionine in schizophrenics is not due to a diversion of methionine into protein synthesis.

The results illustrated in Fig. 5 indicate that the amount of the label appearing in proteins is higher from L-methionine-methyl-^{14}C than from L-methionine-1-^{14}C. To explain these differences the metabolic pathways of L-methionine must be considered (Figs. 9, 10,

and 11). In addition to direct incorporation of L-methionine-methyl-^{14}C, other amino acids labelled by ^{14}C from the methyl group of L-methionine-methyl-^{14}C through the methyl pool may be incorporated into proteins. The pathway for labelling of some of these amino acids such as glycine and serine by the methyl label of L-methionine via choline and betaine is shown in Fig. 11. In the case of L-methionine-carboxyl-^{14}C, however, its label can appear in cellular proteins simply through direct incorporation of carboxyl ^{14}C-labelled methionine into proteins.

Incorporation of the methyl label into proteins from L-methionine was unaltered for leukocytes of controls in presence of chlorpromazine in the medium. In the case of psychiatric patients, on the other hand, leukocytes incorporated significantly smaller amounts of the label from the methyl group into proteins when chlorpromazine was added to the incubation medium (Table 3). The suppression of protein synthesis by the leukocytes in the presence of chlorpromazine may be an important factor in bringing about leukopenia by this drug *in vivo*.[39] It is feasible that synthesis of adequate amounts of proteins vital to the cells may be inhibited by chlorpromazine, eventually causing cell death. Also, leukocytes of different individuals may vary in sensitivity to this tranquillizer. This could explain why in the cells from controls, chlorpromazine did not produce depression in protein synthesis in these experiments. Moreover, the cell death due to depression in protein synthesis by the presence of chlorpromazine in the incubation medium must be gradual, since the cells retain or improve their ability to oxidize methyl carbon atom of L-methionine (patients #13 and 17, Table 3) in spite of the fact that protein synthesis is nearly halved.

The results of Fig. 8 suggest that the doses of chlorpromazine which the cells can tolerate in vitro vary from one individual to the other. This may be significant in deciding on the maximum dose of chlorpromazine that can be given to each patient.

The results presented in this section are indicative of alteration of oxidation of the methyl carbon atom of L-methionine in schizophrenia but not conclusive of the extent and of the absolute significance of the involvement of methylation processes. Predisposition to schizophrenia whether it is monogenically or polygenically determined[40] may indeed not be brought about by a single metabolic defect but by a number of different ones. Whether alteration in oxidation of the methyl carbon atom of L-methionine is one facet of the polymorphism of the metabolic defects or is a common factor to all forms of schizophrenia awaits the study of a large number of acute cases. The fact that alteration in metabolism of the methyl carbon atom of L-methionine has been detected in schizophrenia in vitro not only supports the hypothesis claiming a metabolic basis for schizophrenia but also opens up a new approach to the study of this disease.

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