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Fall 1969

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Fall 1969

SEMIANNUAL REPORT
BIOLOGY AND MEDICINE

John H. Lawrence, M.D., Editor

DONNER LABORATORY AND DONNER PAVILION

LAWRENCE RADIATION LABORATORY UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA

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Foreword

This Semiannual Report again reflects, but does not completely cover, the varied areas of progress in research during the past six months. The studies included, ranging from basic to clinical, re-emphasize the increasing value of isotopes and radiation to research in Biology and Medicine. One of the papers, "Visualization of Radioactivity in the Dog Following Administration of Various C-Carboxylates," is the basis for the award of the first annual George Von Hevesy prize in Nuclear Medicine, presented at the annual meeting of the European Society of Nuclear Medicine, Zürich, September 1969.
Abstract

Visualization of radioactivity in the dog following administration of various $^{13}$C-carboxylates. Markedly increased bone blood flow in myelofibrosis. A case of a typical myeloid metaplasia with myeloid C-trisomy. Iron absorption after portocaval shunt operations. Increased iron absorption in hemochromatosis before and after phlebotomy therapy. Effects of large doses of unlabeled L-methionine on $^{14}$CO$_2$ production in rats given L-histidine (imidazole-2-$^{14}$C), L-serine-3-$^{14}$C, formimino-$^{14}$C L-glutamic acid, and $^{14}$C-formate. Free radicals in pyrimidines: ESR of irradiated single crystals of orotic acid. Irradiation of dihydrothymine in frozen sulfuric acid solutions. Subfractionation of the low-density $S_f$ 0-20 and high-density lipoproteins. The glycoprotein nature of the smooth membranes of acid-secreting cells. Information content of electron microscope images: color and depth information as vehicles for subjective information transfer. Induction of psychotic behavior in folic-acid-deficient patients by ingestion of L-methionine. Dosimetry of $\pi$ mesons using silicon detectors and plastic scintillators. RBE and OER at various points on the modified depth-dose distribution of 910-MeV helium ion beam using cultured cells (T1). RBE and OER of $\pi^-$ mesons for induction of heteroallelic reversion in diploid yeast.
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Visualization of Radioactivity in the Dog Following Administration of Various $^{11}$C-Carboxylates

H. Saul Winchell and Meldrum B. Winstead

SUMMARY

Low-radiation-dose high-resolution tomographic positron scintigraphy, and potential for isologous labeling of a large variety of organic compounds attendant on its use, make $^{14}$C potentially an extremely important radioisotope in nuclear medicine. This work with $^{13}$C-carboxylates demonstrates rapid renal concentration and excretion of benzoate, p-chlorobenzoate, and 2-thiophenecarboxylate, suggesting their usefulness in morphological as well as functional studies of this organ. Hepatic as well as renal concentration of phenylacetate, 3,4-dimethoxybenzoate, o-hydroxybenzoate (salicylate), and cyclohexanecarboxylate suggests their use in morphological and functional studies of both these organs. (The last two compounds appear to show some concentration in the heart as well.) In addition to accumulation and excretion of 1-naphthoate and pentanoate in the liver and kidneys these compounds were localized in the gall bladder, suggesting their use in morphological and functional studies of this organ. The apparent exclusion of all these compounds from the brain and their lack of concentration in salivary glands and nasal sinuses suggest their possible usefulness in visualization of brain lesions. The diffuse abdominal distribution of butyrate and octanoate suggests accumulation in mesenteric and omental fat, and if such is the case, further studies with these materials might be useful in visualizing the kinetics of fatty-acid deposition.

Prior to the general availability of $^{14}$C, the positron-emitting radioisotope $^{11}$C, with a half-life of 20.4 minutes, was the principal radioisotopic tracer of carbon in chemical and biological studies (1). Curie quantities of $^{11}$C were produced and a large number of $^{11}$C compounds were synthesized (1). Unfortunately, devices for imaging the in vivo distribution of radioisotopes in the body were not available at that time, and the virtues of the annihilation $\gamma$ rays arising from the decay of $^{11}$C were insufficient to offset the inconvenience of its short half-life in comparison with $^{14}$C. The present availability of devices for imaging the distribution of positron-emitting radioisotopes within the body has led to renewed interest in $^{11}$C-labeled compounds (2). For example, we expect that $^{11}$C-dopamine would be useful in visualization of the adrenal medulla and for localization of metastases from neuroblastoma and melanoma (3), and that similarly labeled asparagine might be useful in visualizing asparagine-dependent tumors (4).

In an initial effort to develop techniques for the production and rapid incorporation of $^{11}$C into organic compounds, we have synthesized a series of $^{11}$C-labeled carboxylic acids and evaluated the relationship between
their chemical structure and their distribution within the body. The in vivo distribution of these $^{14}$C-carboxylates was visualized, as a function of time, by utilizing a rapid-imaging whole-body scanner and a positron camera equipped with a tomographic attachment allowing for simultaneous visualization of the distribution of radioisotopes at various depths within the body. The results of these studies are presented herein and discussed with regard to the potential clinic usefulness of these compounds.

MATERIALS AND METHODS

Figure 1 represents the method of preparation of the $^{11}$C-carboxylic acids investigated in this study. The $\text{B}_2\text{O}_3$ was fused on a corrugated surface set at 30 deg with respect to the incident beam of charged particles. When deuterons were the bombarding particles, the $\text{B}_2\text{O}_3$ that was used was 90% enriched in the $^{10}$B isotope (20th Century Electronics, Ltd., New Addington, Surrey, England), and the reaction that occurred was $^{10}\text{B}(d, n)^{11}\text{C}$. When protons were the bombarding particles, natural $\text{B}_2\text{O}_3$ containing 80.4% of the $^{11}$B isotope was used, and the reaction was $^{11}\text{B}(p, n)^{14}\text{C}$. When deuteron beam currents were greater than 10 $\mu$A, the isolation or target foils (1-mil Al or 2-mil Pt) usually ruptured, therefore deuteron beam currents were kept below 10 $\mu$A. However, up to 35 $\mu$A of proton beam current could be used without rupture of the isolation or target foils. In each case the deuteron or proton beam was maximally defocused prior to bombardment. The $^{11}\text{C}$ was liberated from the target by recoil primarily as $^{11}\text{CO}$ and $^{11}\text{CO}_2$ and was carried from the target by a gas stream composed of dry nitrogen containing 0.5% carbon monoxide and 0.5% carbon dioxide. The gas stream was passed over a column of Drierite to remove water vapor, and the carbon monoxide was oxidized to carbon dioxide by passage through a 33-cm-long column packed with CuO and heated at 700°C. The gas was then cooled by passage through copper coils immersed in an iced bath. The gas flow rates were held at approximately 0.5 to 1 liter of gas per min.

The stream of gas containing $^{14}\text{CO}_2$ and carrier $^{12}\text{CO}_2$ was passed for 20 to 30 min (or until a maximum activity reading was obtained with a radiation-detection device mounted against the outside of the iced-water bath, see Fig. 1) into a cold solution of 5 to 10 mmoles of either the appropriate Grignard reagent \([\text{R(or Ar)}-\text{MgX}, \text{X}=\text{Br or Cl}]\) or aryl lithium reagent (ArLi) dissolved in 20 to 25 ml of anhydrous ether.
Following the carbonation of the Grignard or aryl lithium reagent, the reaction mixture was hydrolyzed by the addition of 5 ml of 6 N hydrochloric acid. A stream of nitrogen gas was passed through the reaction mixture during both the acid hydrolysis and subsequent bicarbonate extraction, in order to obtain adequate mixing of the reagents.

After removal of the lower hydrochloric acid layer by use of the externally operated solenoid valve, the upper ether layer containing the $^{11}$C-carboxylic acid was extracted with 20 to 25 ml of 6% aqueous sodium bicarbonate. The aqueous bicarbonate layer containing the sodium $^{11}$C-carboxylate was removed and heated to boiling to remove traces of ether. The aqueous solution was then transferred to a serum bottle and sterilized prior to its administration by passing through a millipore filter.

Representative reactions for the preparation of the sodium $^{11}$C-carboxylates listed in Table 1 are illustrated as follows for sodium $^{11}$C-octanoate (reaction 1) and sodium $^{11}$C-naphthoate (reaction 2):

$$
\text{CH}_3\text{(CH}_2\text{)}_6\text{COOMgBr} \xrightarrow{\text{CO}_2} \text{CH}_3\text{(CH}_2\text{)}_6\text{COOH} + \text{MgBr}_2
$$

(1)

$$
\text{CH}_3\text{(CH}_2\text{)}_6\text{COOH} \xrightarrow{\text{NaHCO}_3} \text{CH}_3\text{(CH}_2\text{)}_6\text{COONa}
$$

(2)

Prior to the preparation of each of the $^{11}$C-carboxylates listed in Table 1, experiments using $^{14}$C-labeled carbon dioxide with carrier carbon dioxide demonstrated that from 50 to more than 90% of the $^{14}$C introduced into the Grignard or aryl lithium reagent was converted into the corresponding sodium $^{14}$C-carboxylate. Essentially all the $^{14}$CO$_2$ that was introduced into the system was absorbed in the solution containing the Grignard or aryl lithium reagent, as negligible quantities of radioactivity were recovered in a 2-methoxyethanol-ethanolamine trap placed in series with the system.

Prior to the $^{11}$C and $^{14}$C carbonation reactions, the carboxylic acids listed in Table 1 were prepared by carbonating the appropriate Grignard or aryl lithium reagent with $^{12}$CO$_2$ under experimental conditions similar to those described for the preparation of the radioactive carboxylic acid. The resulting carboxylic acids were isolated and purified, and their yields and the melting points of the crystalline acids were ascertained as a confirmation of their respective values reported in the literature.

The distribution of $^{11}$C-radioisotope within the entire body of the dog following administration of the $^{11}$C-carboxylates was obtained by using the rapid-imaging whole-body scanner previously described by H. O. Anger (Donner Laboratory, University of California, Berkeley, California) (5). Localization of the positron-emitting $^{11}$C within specific regions of the animal was determined by utilizing the positron camera previously described (2) and modified by Anger to obtain tomographic visualization along six different focal planes while simultaneously obtaining standard positron scintiphotos at varying exposures.
WHOLE BODY SCANS FOLLOWING I.V. ADMINISTRATION OF REPRESENTATIVE ¹¹C-CARBOXYL LABELED CARBOXYLIC ACIDS TO DOGS
(Scan following ¹³¹I-albumin administration shown for comparison)

<table>
<thead>
<tr>
<th>Compound</th>
<th>¹³¹I-albumin</th>
<th>Acetate</th>
<th>p-chlorobenzoate</th>
<th>Cyclohexanecarboxylate</th>
<th>Butyrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of scan</td>
<td>(min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>following admin.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10-16</td>
<td>32%-37%</td>
<td>4.6</td>
<td>5%-6</td>
<td>38%-42%</td>
</tr>
</tbody>
</table>

Fig. 2. Whole-body scans following intravenous administration of representative ¹¹C-carboxyl-labeled carboxylic acids to dogs.

RESULTS

Figure 2 presents whole-body scans obtained following intravenous administration of representative compounds synthesized and studied in this work. In the left-hand portion of the figure a whole-body scan obtained during the 10- to 16-min time interval after administration of ¹³¹I-albumin is presented to demonstrate the pattern of distribution of materials limited to the intravascular space. Locations of the heart, liver, kidneys, and bladder are indicated on the figure. On these and subsequent figures the scans represent anterior-posterior views. The scans were performed during the time interval indicated above each scan. The right- and left-hand sides of the body are indicated by R and L, respectively.

Homogeneous whole-body distribution of ¹¹C is demonstrated by the scan obtained 32.75 to 37.5 min subsequent to the intravenous administration of ¹¹C-acetate. Rapid concentration and excretion by the kidney is shown for ¹¹C-labeled p-chlorobenzoate. Early accumulation of ¹¹C activity in the heart, liver, kidneys, and bladder is indicated 5.25 to 6 min after intravenous administration of ¹¹C-cyclohexanecarboxylate. A much larger quantity of this material is present in the bladder 36.5 to 42.5 min after its administration, but there is significantly less activity seen in the region of the heart. Generalized distribution of activity in the entire abdomen without specific organ visualization is shown 2.5 to 3.5 min following intravenous administration of ¹¹C-butyrat.

Figure 3 shows serial whole-body scans following intravenous administration of ¹¹C-pentanoate to a dog. It can be seen that there is a rapid initial accumulation of activity in the liver, with subsequent excretion of ¹¹C activity in the gall bladder. An additional small amount, cleared apparently by the kidneys, is found in the urinary bladder.

The upper scan of Fig. 4 shows a six-lens representation of ¹¹C activity in the liver at varying intensities following the intravenous administration of ¹¹C-naphthoate to a dog. The focal plane was fixed at approximately 2
<table>
<thead>
<tr>
<th>Time of scan following admin. (min)</th>
<th>6½-7½</th>
<th>36-37½</th>
<th>49-60</th>
<th>Positron scintiphoto of $^{14}$C activity in gall bladder</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>123-131½</td>
</tr>
</tbody>
</table>

Fig. 3. In vivo temporal distribution of $^{14}$C following intravenous administration of $^{14}$C-pentanoate to dogs.

XBB 696-3521

Fig. 4. Tomographic positron scintiphoto of liver 74-84.75 min following intravenous administration of $^{14}$C-naphthoate to a dog.

6 lens representation with varying intensity. Focal plane fixed approx. 2 inches below detector head.

Intensity fixed. 6 serial focal planes at 1, 2, 3, 4, 5 and 6 inches below detector head.

In the lower portion of this figure localization of activity in the gall bladder is obvious at focal planes 4, 5, and 6 in. below the detector head. In the lower portion of this figure localization of activity in the region of the gall bladder is obvious at focal planes 4, 5, and 6 in. below the detector head.

in. below the detector head, and from the scintiphotos the localization of activity in the gall bladder is not obvious. The lower portion of this figure illustrates scintiphotos at a fixed intensity with six serial focal-plane settings at 1, 2, 3, 4, 5, and 6 in. below the detector head.
Table 1 summarizes the localization of radioactivity in dogs subsequent to the intravenous administration of various \( ^{14} \text{C} \)-carboxylic acids. \( ^{11} \text{C} \) activity is found homogeneously throughout the whole body subsequent to administration of \( ^{14} \text{C} \)-acetate and 9-phenanthrene-carboxylate. Subsequent to administration of butyrate and octanoate, most of the activity is found to be distributed homogeneously throughout the entire abdomen, whereas subsequent to administration of benzoate, p-chlorobenzoate, and 2-thiophene-carboxylate, localization of \( ^{11} \text{C} \) activity is confined primarily to the kidneys and urinary bladder. When phenylacetate or 3,4-dimethoxybenzoate is administered, significant activity is seen in the liver as well as the kidneys and urinary bladder. After administration of o-hydroxybenzoate (salicylate) and cyclohexanecarboxylate, there appears to be a significant accumulation of activity in the region of the heart, in addition to the liver, kidneys, and bladder. \( ^{11} \text{C} \)-labeled 1-naphthoate and pentanoate show some accumulation of activity in the gall bladder in addition to the liver, kidneys, and urinary bladder.

**DISCUSSION**

Knowledge of the spatial distribution of \( ^{11} \text{C} \)-labeled compounds within the body adds another dimension to our understanding of metabolism of organic compounds. The localization of butyrate and octanoate homogeneously throughout the abdomen suggests that plasma fatty acids are in more rapid equilibrium with fat located within mesentery and omentum than other fat stores throughout the body. Benzoate, p-chlorobenzoate, and 2-thiophene-carboxylate are very rapidly cleared from the body by the kidney. Thus, if any significant conjugation within the liver occurs it must proceed quite rapidly, possibly during a single pass of the material through the liver, since insufficient \( ^{11} \text{C} \) accumulates in the liver at any time to allow for its visualization. The potential usefulness of \( ^{11} \text{C} \)-benzoate in delineation of renal lesions has been discussed in a previous communication (6).

On the other hand, phenylacetate and 3,4-dimethoxybenzoate are concentrated by the liver as well as the kidneys, suggesting that their hepatic metabolism requires a significant time. The results with o-hydroxybenzoate (salicylate) and cyclohexanecarboxylate are similar to those for the compounds just mentioned. However, they appear to have some cardiac localization soon after their administration. Whether this is indeed the case or whether the scans simply represent retention of these materials within the blood remains to be determined. If this latter situation is what is actually being observed, then the radioisotopic cardiac silhouette is merely a reflection of activity in the blood pool. In addition to being concentrated in the liver and kidneys, 1-naphthoate and pentanoate are excreted into the bile, allowing for visualization of the gall bladder. The homogeneous whole-body distribution of activity following administration of \( ^{11} \text{C} \)-labeled acetate or 9-phenanthrene-carboxylate suggests that either the carboxyl group of these compounds is rapidly degraded to bicarbonate or these materials rapidly distribute themselves homogeneously throughout body spaces. Simultaneous kinetic analysis of plasma \( ^{11} \text{C} \) clearance curves could have elucidated these points, but, unfortunately, were not performed in this study.

It is noteworthy that chlorination of benzoic acid at the para position (p-chlorobenzoate) does not alter the spatial distribution of the resulting compound from that seen for benzoate. However, the addition of a carbon atom between the carboxyl group and the benzene ring (phenylacetate) results in markedly greater accumulation of the resulting material in the liver than is observed with benzoate.
### Table I. Localization of radioactivity in the dog following administration of various $^{14}$C-carboxylates.

<table>
<thead>
<tr>
<th>Name</th>
<th>Compound</th>
<th>Structure</th>
<th>Localization of radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate $^a$</td>
<td>$\text{CH}_3^{14}\text{COONa}$</td>
<td>Homogeneous whole body</td>
<td></td>
</tr>
<tr>
<td>9-phenanthrene carboxylate $^b$</td>
<td></td>
<td>Homogeneous whole body $^c$</td>
<td></td>
</tr>
<tr>
<td>Butyrate $^a$</td>
<td>$\text{CH}_3(\text{CH}_2)^{14}\text{COONa}$</td>
<td>Entire abdomen</td>
<td></td>
</tr>
<tr>
<td>Octanoate $^a$</td>
<td>$\text{CH}_3(\text{CH}_2)_6^{14}\text{COONa}$</td>
<td>Entire abdomen</td>
<td></td>
</tr>
<tr>
<td>Benzoate $^a$</td>
<td>$\text{CH}_4^{14}\text{COONa}$</td>
<td>Kidneys, bladder</td>
<td></td>
</tr>
<tr>
<td>p-chlorobenzoate $^a$</td>
<td>$\text{Cl}^{14}\text{COONa}$</td>
<td>Kidneys, bladder</td>
<td></td>
</tr>
<tr>
<td>2-thiophene carboxylate $^d$</td>
<td>$\text{S}^{14}\text{COONa}$</td>
<td>Kidneys, bladder</td>
<td></td>
</tr>
<tr>
<td>Phenylacetate $^a$</td>
<td>$\text{CH}_3\text{COONa}$</td>
<td>Liver, kidneys, bladder</td>
<td></td>
</tr>
<tr>
<td>3,4-dimethoxybenzoate $^e$</td>
<td>$\text{CH}_3\text{O}-\text{CH}_2^{14}\text{COONa}$</td>
<td>Liver, kidneys, bladder (heart?)</td>
<td></td>
</tr>
<tr>
<td>O-hydroxybenzoate $^f$ (salicylate)</td>
<td>$\text{OH}^{14}\text{COONa}$</td>
<td>Liver, kidneys, bladder (heart?)</td>
<td></td>
</tr>
<tr>
<td>Cyclohexanecarboxylate $^a$</td>
<td>$\text{CH}_4^{14}\text{COONa}$</td>
<td>Liver, kidneys, bladder (heart?)</td>
<td></td>
</tr>
<tr>
<td>1-naphthoate $^g$</td>
<td>$\text{CH}_3(\text{CH}_2)_2^{14}\text{COONa}$</td>
<td>Liver, gall bladder, kidneys, bladder</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ This compound was prepared by carbonating the appropriate Grignard reagent, which was obtained from either Arapahoe Chemicals, Boulder, Colorado, or Alfa Inorganics, Inc., Beverly, Massachusetts.

$^b$ This compound was prepared by carbonating 9-phenanthryllithium, which was prepared from 9-bromophenanthrene and n-butyl lithium [H. Gilman and T. H. Cook, J. Am. Chem. Soc. 52, 2813 (1930)].

$^c$ During the initial 10 min. whole-body scans were similar to those obtained with $^{125}$I-albumin. After 2 hours whole-body scans demonstrated an accumulation of radioactivity in the right upper quadrant of the abdomen.

$^d$ This compound was prepared by carbonating 2-thienylmagnesium bromide, which was prepared from 2-bromothiophene and magnesium [D. A. Shirley, Preparation of Organic Intermediates (John Wiley & Sons, New York, 1951), p. 282].

$^e$ This compound was prepared by carbonating, at 60°C, 3,4-dimethoxyphenyl lithium, which was prepared from 4-bromoveratrole and n-butyl lithium [M. Calvin, C. Heidelberger, J. C. Reid, B. M. Tolbert, and P. F. Yankwich, Isotopic Carbon (John Wiley & Sons, New York 1949), pp. 183 and 184].

$^f$ This compound was prepared by carbonating the aryl lithium intermediate, which was obtained from the reaction of o-bromophenol with n-butyl lithium [H. Gilman and C. E. Arratier, J. Am. Chem. Soc. 59, 1537 (1938)].

$^g$ This compound was prepared by carbonating 1-naphthyllithium, which was prepared from 1-bromonaphthalene and n-butyl lithium [H. Gilman and F. W. Moore, J. Am. Chem. Soc. 52, 1843 (1930)].
Similar results are obtained by the addition of methoxy groups at the 3 and 4 positions of benzoate.

It is also noteworthy that the naturally occurring butyrate and octanoate are not significantly excreted by the liver or kidneys, but the odd-number carbon atom aliphatic fatty acid pentanoate, which is normally not found within the body, is concentrated in the liver and kidneys and excreted in the bile and urine.

Positron scintigraphic tomography shows promise of being clinically useful, as can be appreciated from Fig. 4, and discussions of the technique will be published in the future by H. O. Anger. The apparent exclusion of these $^{11}$C-carboxylates from the brain and their failure to concentrate in salivary glands or nasal sinuses suggest their possible usefulness in delineation of brain lesions.

ACKNOWLEDGMENTS

The authors thank Dr. W. G. Myers of the Ohio State University, Columbus, Ohio, for his encouragement and suggestions concerning cyclotron production of $^{11}$C. We are grateful also for the assistance of Dr. R. Fawwaz and G. Armaly of the Lawrence Radiation Laboratory.

REFERENCES AND NOTE


This paper reports some of the investigations that resulted in the award of the first annual George von Hevesy prize to Dr. Winchell and Dr. Winstead.
Markedly Increased Bone Blood Flow in Myelofibrosis

Donald C. Van Dyke, Hal O. Anger, Howard G. Parker, James McRae, Ernest L. Dobson, Yukio Yano, Jean-Pierre Naets and John A. Linfoot

SUMMARY

The rate of appearance of an intravenously administered dose of $^{18}$F in the skeleton is a function of the size of the skeletal blood supply. $^{18}$F scintigraphic and kinetic data have shown a marked increase in skeletal blood flow in patients with myelofibrosis, both idiopathic and secondary to polycythemia vera. There was a clear-cut increase in the "minimum bone blood flow" from a mean value of 3.3% of cardiac output in the control group to 7.8% in patients with myelofibrosis. That the skeleton shows striking morphologic abnormalities in advanced cases of myelofibrosis is well known, but that the pathology is associated with an increase in bone blood perfusion comparable to that seen in generalized Paget's disease of bone has not previously been suspected.

The fact that three of the patients with myelofibrosis and markedly increased bone blood flow were not anemic (two with compensatory myeloid metaplasia and one in the active stage of polycythemia vera) rules out the possibility that the abnormality is secondary to anemia, per se. The hypervascularity is then either primary or secondary to the poorly understood medullary disease.

That the skeleton is markedly abnormal in advanced cases of myelofibrosis has been known since Vaughan and Harrison's well-documented study (1), and this is re-emphasized each time the clinician examines a bone biopsy from such a case or observes the sometimes strikingly abnormal roentgenograms. That the skeletal pathology is associated with an increase in bone blood perfusion such as that seen in Paget's disease of bone has not previously been suspected.

The rate of appearance of $^{18}$F in the skeleton is principally determined by skeletal blood perfusion, making it possible to investigate the bone blood flow associated with diseases involving bone and bone marrow. The evidence and assumptions involved in interpreting skeletal uptake of $^{18}$F ion as a function of bone blood perfusion rate have been presented previously (2). The distribution of positron-emitting isotopes such as $^{18}$F in the human body can be accurately visualized by using the positron scintillation camera (3) and a specially designed whole-body scanner (4). In this study scintigraphic and fluoro-kinetic data have been obtained showing a marked increase in skeletal blood flow associated with myelofibrosis, both idiopathic and secondary to polycythemia vera.

MATERIALS AND METHODS

Well-documented cases of myelofibrosis were chosen for complete fluorokinetic studies, but the duration, severity, and extent of extramedullary hematopoiesis varied from...
those recently developed to others of long duration, completely compensated or requiring transfusions for maintenance of red cell count. Diagnosis was established by bone and marrow biopsy, by $^{52}$Fe whole-body marrow distribution studies (5), and by ferrokinetic studies in some cases. Controls were volunteers, patients with blood conditions other than myelofibrosis, or hematopoietically normal subjects with various bone diseases.

**Scintigraphy**

Scintigraphy using the positron scintillation camera, the whole-body scanner, Mark II, and the method of preparation of $^{18}$F are described elsewhere (3, 4, 6).

The subjects were placed on the bed of the whole-body scanner, a plastic needle was inserted into the vein of one arm for blood sampling, 600 $\mu$Ci of $^{18}$F was given intravenously in the opposite arm, and scans were made at frequent intervals thereafter. At the end of a 3-hr period urine was collected to determine renal excretion of $^{18}$F. Completeness of voiding was determined by taking a scan immediately after the urine collection. In some patients (see below) blood samples were taken frequently during the scanning procedure for 3 hr after $^{18}$F injection. A graph of the activity profile was produced simultaneously with the whole-body scan.

**Fluorokinetic analysis**

Blood samples were taken frequently and urine was collected at 3 hr to determine renal excretion of $^{18}$F during this period. The blood data were fitted to a two-component curve (Fig. 1). Blood and urine data were analyzed by using two different physiologically plausible two-compartment systems (Fig. 2). Without measuring the fraction of the injected dose in bone as a function of time, it is not possible to choose clearly which one gives a better value for bone blood flow, except as experience indicates which seems more reasonable. The second kinetic model presented in Fig. 2 results in a value about three times that of the first. However, regardless which of the two is chosen, a good value for renal $^{18}$F clearance could be obtained by relating the blood level over the 3-hr period to the urinary excretion. In addition, the total clearance of $^{18}$F was obtained from the area under the blood curve, and the extrarenal...
clearance by subtracting renal clearance from the total.

Compartment-system analysis has been based on the counting rate of $^{18}$F in whole blood. From evidence that there is rapid exchange of $^{18}$F between plasma and red cells, and lack of protein binding of $^{18}$F in blood within the 3- to 4-hr period of the measurements, values in whole blood rather than plasma seem the best initial choice to relate to the kinetic model.

Analysis has been based on the two-compartment models. A single-compartment model (exchangeable fluoride pool) is clearly not adequate to explain the curves obtained. Comparison of blood curves with whole-body scans and counts over bone give strong indications that at least a three-compartment system would be necessary to fully reflect the striking changes so evident in the scans: an initial mixing volume, a larger space with which $^{18}$F can equilibrate more slowly, and a labile pool in bone. However, unless one can sample in at least one of these other compartments, analysis of the system would have to depend on various arbitrary assumptions about exchange rates and pool sizes. We are therefore continuing to investigate how much can be learned from the simpler two-compartment analysis.

It appears reasonable thus far to equate extrarenal clearance of $^{18}$F with "minimum bone blood flow," the value for bone blood flow from model A, Fig. 2. This is the value for bone flow if bone is 100% efficient in removing $^{18}$F from the circulation and releases none of it during the period of observation. This minimum value for bone blood flow, like the renal clearance and total clearance, is actually independent of the number of compartments and their arrangement, so long as the area under the curve is accurately approximated. Bone blood flow values thus obtained are comparable with minimum values obtained by us in small animals and by others with $^{85}$Sr and $^{45}$Ca, making rather similar assumptions, and in some cases measuring bone uptake directly (7, 8).

**Metabolic studies**

In order to evaluate calcium and bone metabolism in patients with myelofibrosis, five were placed on a low-phosphate (340 mg) diet for 3 days during which time daily 24-hr urine collections and appropriate blood samples were obtained to measure blood and urine calcium, serum and urine phosphorus, serum creatinine, creatinine clearance, phosphate clearance, and tubular reabsorption of phosphate (TRP). In addition, 4-hr calcium infusions were performed according to the method of Bhandarkar and Nordin (9). Endocrine studies included PBI and T-3 resin uptake, plasma and urinary 11-oxycorticosteroid (10), and growth hormone measurements following arginine and insulin stimulation. Growth hormone assays were performed by radioimmunoassay using the technique of Garcia et al. (11). The arginine and modified insulin tolerance tests were performed using the method previously described (12).

**RESULTS**

**Scintigraphy**

In the course of these studies of patients with myelofibrosis and of hematopoietically normal subjects, it became apparent that the most striking difference in appearance of the scans occurred 10 to 40 min after injection of the isotope. This follows from the fact that immediately after injection the distribution is essentially uniform in all subjects and at long times after injection the only appreciable $^{18}$F concentration remaining is in the bone in all subjects. The only difference, then, is one
Fig. 3. Markedly increased bone blood flow in patient (M. M.) with myelofibrosis (A), as demonstrated with $^{18}$F and the Donner Laboratory Mark II whole-body scanner. The subjects were each given 600 µCi of $^{18}$F and the scan started 15 min later (scan duration: 22 min). In the normal subject (B) the kidneys, bladder, and general body outline are visible but the skeleton has not yet accumulated sufficient isotope to be clearly evident. In patients with myelofibrosis the skeleton is clearly evident within a few minutes after injection. Skeletal clearance of the isotope is so rapid that renal clearance is relatively small.

XBB 697-4550

Fig. 4. Mark II whole-body scan with four images taken simultaneously of distribution of $^{18}$F in a normal subject (A) and a patient with myelofibrosis and agranocytic myeloid metaplasia secondary to polycythemia vera (B). The skeleton was clearly evident in the patient with myelofibrosis 15 min after intravenous injection of 600 µCi $^{18}$F (scanning time: 22 min).

XBB 6910-6823 XBB 696-4050

of absolute intensity, which is difficult to judge visually in a scan. In the mid-time period, however, a comparison between bone and soft tissue concentration can readily be made by visual observation. The illustrations presented in Figs. 3 and 4 were made by taking scans starting 15 min after $^{18}$F injection. Each scan illustrated required 22 min. Shorter scans were taken at intervals during a 3-hr period. In some cases, high-resolution positron scintillation camera pictures of $^{18}$F distribution were taken as well, Fig. 5. The Donner whole-body scanner takes four images simultaneously, only one of which is shown in Fig. 3 for the sake of simplicity. The composite readout is shown in Fig. 4. In normal subjects at this time (14-37 min), the skeleton has not yet accumulated enough isotope to be clearly visible, the distribution of isotope in soft tissues outlines the entire
Fig. 5. Comparison of whole-body scan and positron camera pictures of patient J. D. 3 hr after $^{18}$F injection. The figure compares the resolution of the two instruments and illustrates the degree of involvement of peripheral skeleton in this patient.

$^{52}$Fe scanning, Fig. 6, but was well compensated by extramedullary erythropoiesis in a very large spleen. She did not require transfusions and had no evidence of renal disease. The diagnosis was agnogenic myelofibrosis with myeloid metaplasia.

The 65-year-old female patient (E. K.) shown in Fig. 4B developed myelofibrosis and agnogenic myeloid metaplasia secondary to polycythemia vera. She was maintained by transfusion and was uremic (BUN: 34 mg%).

Figure 5 compares whole-body scan and positron camera pictures of $^{18}$F distribution in patient J. D., a 60-year-old man with idiopathic myelofibrosis and myeloid metaplasia. Pictures were taken 3 hr after injection of the isotope, when skeletal uptake and renal excretion had reduced the amount of isotope in soft tissues to near background. The higher-resolution camera pictures of the hand show the degree of involvement of many of the small peripheral bones in this patient.

In order to determine whether a generalized increase in bone blood flow occurred only in myelofibrosis, patients with other blood diseases as well as patients with bone disease but not evidence of disease involving the marrow were studied, Table I. The table includes the 12 patients on whom complete fluorokinetic analysis was made as well as 25 classified by simple visual comparison of scans (22-min scan started 15 min after injection of $^{18}$F). Since some degree of myelofibrosis is found in many patients with polycythemia vera (13), the distinction made in the table was based on whether the patient currently required phlebotomy or myelosuppressive therapy (polycythemia vera) or was in the "spent" phase requiring no therapy or transfusion (myelofibrosis secondary to polycythemia vera).
Table I. Gross evaluation of skeletal blood flow under various conditions.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Total skeletal blood flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myelofibrosis, idiopathic, 4 cases</td>
<td></td>
</tr>
<tr>
<td>Myelofibrosis, secondary to polycythemia vera, 5 cases</td>
<td></td>
</tr>
<tr>
<td>Polycythemia vera, 4 cases</td>
<td></td>
</tr>
<tr>
<td>Autoimmune hemolytic anemia, 1 case</td>
<td></td>
</tr>
<tr>
<td>Congenital spherocytosis, 1 case</td>
<td></td>
</tr>
<tr>
<td>Congenital red cell aplasia, 1 case</td>
<td></td>
</tr>
<tr>
<td>Panhypoplasia, 2 cases</td>
<td></td>
</tr>
<tr>
<td>Chronic myelogenous leukemia, 2 cases</td>
<td></td>
</tr>
<tr>
<td>Lymphoma, 1 case</td>
<td></td>
</tr>
<tr>
<td>Hodgkin's disease, 1 case</td>
<td></td>
</tr>
<tr>
<td>Arterio-venous fistula, 1 case</td>
<td></td>
</tr>
<tr>
<td>Broken hip, 1 case</td>
<td></td>
</tr>
<tr>
<td>Hip pain, 3 cases</td>
<td></td>
</tr>
<tr>
<td>Osteoporosis, 1 case</td>
<td></td>
</tr>
<tr>
<td>Hyperparathyroidism, 1 case</td>
<td></td>
</tr>
<tr>
<td>Acromegaly, 3 cases</td>
<td></td>
</tr>
<tr>
<td>Normal volunteer, 1 case</td>
<td></td>
</tr>
<tr>
<td>Generalized Paget's disease bone, 3 cases</td>
<td></td>
</tr>
<tr>
<td>Metastatic carcinoma, 1 case</td>
<td></td>
</tr>
</tbody>
</table>

a. Not including limited areas of increase due to local disease.

general features are confirmed by a number of less complete sample collections obtained from other subjects and analysed in less elaborate fashion in the preliminary stages of the study.) There was a clear-cut elevation in the "minimum bone blood flow" from a mean value of 3.3% of cardiac output in the control group to 7.8% in the patients with myelofibrosis. The mean value for renal $^{18}$F clearance
Fig. 7. Some selected parameters of interest from fluorokinetic analysis of blood and urine data. Patients with myelofibrosis are represented by solid circles and the control group by open squares. Extrarenal clearance or "minimum bone blood flow" was clearly elevated in the myelofibrotic patients. Urinary excretion of $^{18}$F was reduced not because of reduced renal fluoride clearance, but reflecting competition from bone. The patients represented by the crosses are the cases of far-advanced generalized Paget's disease of bone (Table I).

Fig. 8. Blood disappearance curves of $^{18}$F. Patients with myelofibrosis are represented by solid curves, and those in the control group by dashed curves. The patients represented by the crosses are the cases of Paget's disease, and are not included in the average values from fluorokinetic analysis given in the text.

Don't show any significant difference between the two groups (Fig. 8), but in the fluorokinetic analysis this difference combines with the difference in urinary excretion to result in a rather marked difference in bone blood flow, more nearly in keeping with the dramatic result seen on the scans. A three- or more-compartment model that could take into account competition between bone $^{18}$F adsorption, urinary excretion, and general leakage into and return from extracellular fluid space would perhaps show an even more marked difference between the normal and myelofibrotic bone blood flow, as the scans suggest. Preliminary efforts to quantitate...
the scans indicate about the same values for bone blood flow as analysis of blood and urine activity, but there are unsolved problems in selecting representative areas from the scans, subtracting soft tissue background, etc. Thus while there is general quantitative confirmation, simple visual comparison of scans is at present the most impressive and perhaps the most useful demonstration of the skeletal lesion.

Metabolic studies

The short-term balance studies were performed while most of the patients were on androgen therapy for myelofibrosis. The thyroid function was considered normal in all these patients when changes in binding proteins produced by gonadal steroids were considered. Plasma and urinary adrenal steroids were also normal. The growth hormone responses to arginine or insulin indicate a completely normal growth hormone response. Three of the five patients showed mild degrees of hypocalcemia, with serum calcium values ranging from 8.2 to 8.8 mg/100 ml. Hypocalciuria was present in all patients. One of the patients had mild hypoalbuminemia. Serum albumin was normal in the other patients but two of the four had hyperglobulinemia. Serum phosphorus measurements were slightly low or in the low normal range in all subjects but one (M. M.). Tubular re-absorption of phosphorus was normal in all patients and increased during phosphate restriction, reflecting a normal parathyroid response in these patients. The 4-hr calcium retention tests were abnormal in all patients, indicating a greater avidity for calcium than normal, which parallels the increased fluoride uptake in these patients.

DISCUSSION

The dominant microscopic features of the bone changes in far-advanced myelofibrosis are resorption of bone, altering the compacta into spongy bone, and formation of numerous small trabeculae in the spongiosa on the inner surface of the cortex and throughout the medullary cavity. These alterations combine to increase the bone surface many times. One might suppose that the greatly increased surface could account for the rapid accumulation of $^{18}F$. However, the extraction efficiency of normal bone is virtually 100% and no additional accumulation of $^{18}F$ can occur unless there is greater blood flow past the vastly increased surface. Thus the greatly increased surface must be accompanied by a greatly increased capillary supply.

The fact that three of the patients in this study showing markedly increased bone blood flow were not anemic (two with compensatory myeloid metaplasia and one in the active stage of polycythemia vera) rules out the possibility that the abnormality is secondary to anemia, per se. The hypervascularity is then either primary or secondary to the poorly understood myeloproliferative process. Prolonged severe anemia from causes other than myelofibrosis is frequently associated with redistribution of bone blood flow which accompanies redistribution of marrow (16). A generalised increase in total skeletal flow has been observed in one case of autoimmune hemolytic anemia and one case of congenital spherocytosis, but not in all patients with severe anemia. Presumably those blood diseases which are characteristically associated with roentgenographic changes in bone (14) can be expected to show fluorokinetic changes.

Because of the generalized involvement of the skeleton in myelofibrosis, an abnormality of the endocrine system in influencing bone metabolism was considered. Growth hormone concentration in serum of seven patients with severe myelofibrosis was within normal limits and growth hormone response to insulin-
induced hypoglycemia (12) was not impaired. Adrenal and thyroid function was also normal. Increased calcium retention in all these patients resembled that described for osteomalacia and was quite distinct from the pattern usually seen in hyperparathyroidism, osteoporosis, Paget's disease, and other primary metabolic bone diseases. Parathyroid function was apparently normal, but some degree of secondary hyperparathyroidism could not be excluded. There is considerable recent interest in the role played by bone in marrow function (17–20). It is possible that the bone lesion is primary and that the grossly abnormal bone loses its ability to support hematopoietic marrow. If such is the case, attempts to determine the etiology and direct therapeutic management toward reversal of the bone lesion may lead to better results than restricting attention to the medullary aspects of the lesion. In a recent study of the response of one of the subjects to endosteal curettage (18), failure of curettage to induce an increase in $^{18}$F uptake was unexplained. At the time of that report it was not realized that bone blood flow was abnormally high prior to surgical intervention and that a further increase in response to trauma was apparently not possible.

The possibility that changes in marrow function play an important part of the pathogenesis of age-related osteoporosis has been proposed (21). Agents which affect other bone diseases, e.g., calcitonin (22, 23) and mithramycin (24, 25), should be considered for therapeutic trial in myelofibrosis.

ACKNOWLEDGMENTS

The authors wish to thank David G. Nathan, M. D., Chief, Division of Hematology, Children's Hospital Medical Center, Boston, Massachusetts, for referring two of the patients reported (including patient M. M.), the diagnosis of whom was established through training grant TI AM 05581.

This work was supported in part by the United States Atomic Energy Commission and in part by grant #5ROICA0830 from the National Cancer Institute of the National Institutes of Health.

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A Case of Atypical Myeloid Metaplasia with Myeloid C-Trisomy

William D. Loughman and H. Saul Winchell

SUMMARY

A case of atypical myeloproliferative disorder is presented. Associated with the clinical syndrome is a chromosomal abnormality, C-trisomy, in metaplastic myeloid cells. The abnormality is not found in the patient's lymphocytes. The clinical and cytogenetic findings are compared to those of seven other cases previously reported. The authors agree with a suggestion made in an earlier study, that a form of myeloproliferative disorder associated with C-trisomy may be a distinct clinical entity. The authors suggest collecting additional case data to prove or disprove the contention.

Winkelstein et al. have suggested that some forms of atypical myeloproliferative disorder accompanied by myeloid C-trisomy may represent a distinct clinical entity (1). They present a case, and compare it with two others culled from their review of 22 cases of C-trisomy in the literature. In addition, Kiossoglou et al. earlier had reported C-trisomy in four patients with myelofibrosis and myeloid metaplasia, including two atypical and difficult-to-classify cases (2). In this communication we describe an additional case of atypical myeloproliferative disorder with myeloid C-trisomy, and compare it with the cases cited above, which we review here.

BRIEF CLINICAL SUMMARY

G. N., a white 55-year-old male with a 1-year history of progressive asthenia, was found to have anemia and splenomegaly in October 1967. His bone marrow biopsy was fibrotic and the peripheral blood smear showed morphological changes characteristic of myeloid metaplasia. When seen in this Laboratory in November 1967, his hemoglobin was 9 g%, hematocrit 28.5, and platelets 52,000/mm³, and he showed a mild leukocytosis with a left shift accompanied by circulating normoblasts. Leukocyte alkaline phosphatase was elevated, with a score of 272. Iron kinetics studies in January and October 1968 showed decreased red cell volume, normal plasma volume, normal plasma iron concentration, and increased plasma radioiron clearance rate, and subnormal incorporation of radioiron into circulating red cells. There was a minimal initial accumulation of radioiron in the liver and sacral marrow, with subsequent complete release from sacral marrow but not liver. There was marked initial increase in radioiron in the spleen, with a modest diminution in counting rate in subsequent days. A whole-body scan of erythropoietic sites following ⁵⁹Fe administration showed primary accumulation of radioiron in vertebral bodies and spleen, with some in the liver. There was subsequent virtual complete release of radioiron from marrow cavities, but not from liver or spleen.
In January 1968, the patient was started on high doses of androgens, folic acid, and pyridoxine. Despite this therapy, the patient's hemoglobin dropped to 5.8% by the end of February, necessitating several transfusions in March. His hemoglobin continued to drop to 5.2% by the end of April, and marked increase in spleen size was noted. Multiple transfusions to raise the hemoglobin level were given in May, followed by experimental endosteal curretage of the left femur. Subsequent to this procedure, the patient's hemoglobin slowly dropped from the transfusion-related values, but stabilized at 7 to 8 g% from June to October 1968. During November 1968, the patient noted swelling in ankles, elbows, thighs, and the volar surface of the forearms. Because of a prior history of elevated uric acid levels, the patient was given allopurinol and blood was drawn for latex fixation tests and for Lupus Erythematosus preparations (L-E preparation). In mid-November, the latex fixation test was weakly positive, and the L-E preparation was strongly positive. Previous L-E preparations in October had been negative. Three weeks after allopurinol therapy was instituted the patient's hemoglobin rose to 8.7 g%, and he showed marked symptomatic improvement.

In summary, the patient had the classical peripheral blood and physical examination findings of agnogenic myeloid metaplasia. However, there was atypical retention of active erythropoiesis in vertebral bodies and lack of peripheral movement of marrow. There was ineffective splenic erythropoiesis, most of the patient's circulating hemoglobin being produced in the vertebral bodies.

CYTOGENETIC STUDIES

In June 1968, a routine attempt to culture peripheral white blood cells failed; there was no cellular transformation, and no dividing cells at any time in a 4-day period. Previous experience with similar cases in this Laboratory suggested performance of a repeat study using peripheral blood white cells which had been washed prior to establishing cultures both with and without mitogenic stimulus. In July 1968, white blood cells were thoroughly washed in medium, and replicate cultures were established in NCTC 109 + 10% fetal bovine serum without antibiotics. Half the cultures were provided with phytohemagglutinin (PHA) as a mitotic stimulant; the rest received none. All cultures were examined for cellular morphology, transformation rate, and mitotic index, at zero time and daily for 3 days. Chromosome preparations were made as squashes in 2% lactic-propionic orcein after 5 hours' exposure to a spindle poison (Velban) at each observation time. In hypotonic swelling of cells, and making of permanent preparations, modifications of standard methods (3, 4) were used. The distribution of chromosomes per cell was recorded from counts of 50 or more metaphase cells at each time period, except zero time when only five dividing cells were seen in an estimated 10,000 cells. Photographic karyotypes were made from selected cells at 24, 48, and 68 hours of culture. In unstimulated cultures at 0, 24, and 48 hours, there were very rare transformed cells. After 5 hours' exposure to a spindle poison, the mitotic index of these cells at these sampling times was not higher than 0.2%, and usually much less. The modal number of chromosomes was 47, visually interpreted as C-trisomy. Cells with fewer than 47 chromosomes showed random chromosome loss. Contrasting with results obtained in unstimulated cultures, the frequency of transformed cells in PHA-stimulated cultures rose slowly to about 10% by 68 hours. Dividing cells were first seen at that time with a mitotic index, after 5 hours' exposure to Velban, of only 1.6% of viable mononuclear cells. The modal chromosome number was
46, visually interpreted as normal male diploid. Cells with fewer chromosomes showed random chromosome loss. The PH¹ chromosome, commonly found in many cases of chronic myelogenous leukemia, was not found in any cells of any culture. Karyotypes prepared from photographs of 47-chromosome cells in unstimulated cultures, and 46-chromosome cells in PHA-stimulated cultures, confirmed the visual analysis: eleven 47-chromosome karyotypes showed simple C-trisomy; six 46-chromosome karyotypes showed normal diploidy. Chromatid gaps were slightly elevated over our normal values, but breaks and other aberrations were apparently absent.

Representative karyotypes of both cell types are shown in Figs. 1 and 2, and a summary of the salient features of cytogenetic analysis is given in Table 1.

We regularly find sex chromatin bodies in interphase nuclei of some blast-like cells and "drumstick" appendages on some granulocyte nuclei from marrow and WBC cultures obtained from patients known to be females. In this patient no sex chromatin bodies were found in nuclei of 200 blast-like cells from unstimulated cultures at zero time and 24 hours.

Fig. 1. Karyotype from 47-chromosome cell obtained from unstimulated WBC culture at 24 hr, showing C-trisomy in a presumed metaplastic myeloid cell. Note 16 chromosomes (C-trisomy) in C+X group, with extra chromosome tentatively identified as #10. Scale is 5 microns.

Fig. 2. Karyotype from 46-chromosome cell obtained from a PHA-stimulated WBC culture at 68 hr, showing a normal diploid male chromosome pattern in a presumed lymphocyte. Note 15 chromosomes in C+X group (normal). Scale is 5 microns.
Table I. Summary of chromosome counts and analyses.

<table>
<thead>
<tr>
<th>Time of observation and type of culture</th>
<th>Chromosomes/cell</th>
<th>Number of cells observed</th>
<th>Direct visual analysis</th>
<th>Karyotype modal cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr; unstimulated</td>
<td>&lt; 46</td>
<td>5</td>
<td>...</td>
<td>C-trisomy none made</td>
</tr>
<tr>
<td></td>
<td>46</td>
<td>5</td>
<td>...</td>
<td>C-trisomy</td>
</tr>
<tr>
<td></td>
<td>47</td>
<td>5</td>
<td>...</td>
<td>C-trisomy</td>
</tr>
<tr>
<td></td>
<td>&gt; 47</td>
<td>5</td>
<td>...</td>
<td>C-trisomy</td>
</tr>
<tr>
<td>24 hr; unstimulated</td>
<td>1</td>
<td>7</td>
<td>37</td>
<td>Pseudodiploid; C-trisomy</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>7</td>
<td>37</td>
<td>C-trisomy (2/2)</td>
</tr>
<tr>
<td>48 hr; unstimulated</td>
<td>1</td>
<td>8</td>
<td>40</td>
<td>Pseudodiploid; C-trisomy</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>8</td>
<td>40</td>
<td>C-trisomy (2/2)</td>
</tr>
<tr>
<td>68 hr; PHA stimulus</td>
<td>7</td>
<td>92</td>
<td>100</td>
<td>Random loss Diploid</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>92</td>
<td>100</td>
<td>Diploid (6/6)</td>
</tr>
</tbody>
</table>

a. Poorly visualized, but extra chromosome(s) not in groups A, B, D, F, or G + Y; no polyploidy.

b. Large "marker" chromosome, unlike any member of normal karyotype set.

"Drumsticks" were absent from nuclei of 500 mature granulocytes. Since a proportion of interphase cells with two X chromosomes will show sex chromatin bodies, and a proportion of granulocytes with two X chromosomes will have "drumstick" nuclear appendages, we conclude that the extra C-group chromosome is not an X. Kiossoglou et al., on similar grounds, reach the same conclusion in their studies.

Chromosome pairing is difficult and unreliable within the C group. However, from attempts to create pairs within this group, we believe that the extra chromosome in 47-chromosome cells is one of the medium-sized members of the group, probably similar to #9 and #10 and probably not smaller than #11. This is similar to the tentative identifications made by Winkelstein et al. (1) and Kiossoglou et al. (2).

DISCUSSION

In our experience, PHA often suppresses division in cultures or peripheral WBC of predominantly myeloid origin, such as may be found in some myelogenous leukemia patients with dividing cells present in peripheral blood. On the other hand, it is established that PHA induces only lymphocytes in cultures of peripheral blood to divide (5, 8), and dividing cells are usually seen with low frequency before about 60 hr, and with high frequency for several days after (6, and our own unpublished observations). From these facts, and our culture observations, we conclude that the dividing cells seen in this patient's unstimulated cultures at 0, 24, and 48 hr were myeloid in origin. Since they were found in circulating peripheral blood, they probably represent the metaplastic population of myeloid cells. An attempt to confirm this conclusion by observation of sacral marrow cells failed. Only a few dividing cells with clumped chromosomes were seen on direct observation, and no dividing cells were found in cultures of the biopsy material.

Table 2, patterned after that provided by Winkelstein et al. (1), compares the clinical and cytogenetic findings on our patient with those previously published. Strong similarities in all eight cases are evident, along with some dissimilarities. We concur in the conclusions reached by Winkelstein et al.: "Although it is appealing to consider these patients as representing a separate, distinct clinical entity, such a conclusion appears unjustified at present." However, we feel the similarities are sufficient to warrant both suspicion and the accumulation and presentation of case
Table II. Comparison of eight cases of myeloproliferative disorder and myeloid C-trisomy.

<table>
<thead>
<tr>
<th>Age and sex</th>
<th>Sandberg et al. (7)</th>
<th>Nowell and Hungerford (8)</th>
<th>Winkelstein et al. (1)</th>
<th>Kiossoglou et al. (2)</th>
<th>This case</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age and sex</td>
<td>69, female</td>
<td>55, male</td>
<td>77, female</td>
<td>62–76, 2 male, 2 female</td>
<td>55, male</td>
</tr>
<tr>
<td>Findings on presentation</td>
<td>Anemia; thrombocytopenia</td>
<td>Anemia; thrombocytopenia</td>
<td>Anemia; thrombocytopenia</td>
<td>Not stated</td>
<td>Anemia; thrombocytopenia</td>
</tr>
<tr>
<td>Spleen</td>
<td>Enlarged</td>
<td>Enlarged</td>
<td>Enlarged</td>
<td>Slight to marked enlargement</td>
<td>Enlarged</td>
</tr>
<tr>
<td>Splenic pathology</td>
<td>Myeloid metaplasia</td>
<td>Myeloid metaplasia</td>
<td>Myeloid erythropoiesis, ineffective; splenic sequestration</td>
<td>Not stated</td>
<td>Myeloid erythropoiesis, ineffective; splenic sequestration</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>10.2–10.6</td>
<td>Not stated</td>
<td>5.2–8.7</td>
<td>Not stated</td>
<td>5.2–9.0</td>
</tr>
<tr>
<td>White count</td>
<td>36 000–74 000 (12 200)</td>
<td>Not stated</td>
<td>4 300–236 000</td>
<td>8 400–20 800</td>
<td>6 900–18 500</td>
</tr>
<tr>
<td>Differential white count</td>
<td>Myeloid immaturity; 2% blasts and promyelocytes</td>
<td>Myeloid immaturity; 1–44% blasts</td>
<td>Myeloid immaturity; WBC with occasional blasts</td>
<td>Not stated</td>
<td>Myeloid immaturity; 1–2% blasts; rare dividing cell</td>
</tr>
<tr>
<td>Platelets</td>
<td>54 000–60 000</td>
<td>Not stated</td>
<td>34 000–43 000</td>
<td>Not stated</td>
<td>7 000–52 000</td>
</tr>
<tr>
<td>Reticulocytes</td>
<td>16%</td>
<td>Not stated</td>
<td>16–24%</td>
<td>Not stated</td>
<td>3.2–17%</td>
</tr>
<tr>
<td>Nucleated RBC</td>
<td>3–29 per 100 WBC</td>
<td>Not stated</td>
<td>0–21 per 100 WBC</td>
<td>2–33 per 100 WBC</td>
<td>1–15 per</td>
</tr>
<tr>
<td>Max. no. NRBC/mm³</td>
<td>21 460</td>
<td>Not stated</td>
<td>49 300⁴</td>
<td>Not stated</td>
<td>2 655</td>
</tr>
<tr>
<td>Uric acid</td>
<td>6.2%</td>
<td>Not stated</td>
<td>Normal</td>
<td>8.3%</td>
<td>9.0%</td>
</tr>
<tr>
<td>LAP</td>
<td>Elevated</td>
<td>Normal</td>
<td>Markedly reduced</td>
<td>Reduced in the two atypical; Elevated in the two typical</td>
<td>Elevated</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>Hypocellular; myeloid predominance; erythroid decreased; no megakaryocytes</td>
<td>Hypercellular; (myeloid predominance)</td>
<td>Hypercellular; myeloid and erythroid increased; megakaryocytes decreased</td>
<td>Fibrotic in the typical two; Hypocellular in the two atypical</td>
<td>Hypocellular or fibrotic in sacrum and femur; erythroid slightly increased; Vertebral bodies fully functional</td>
</tr>
<tr>
<td>Duration of illness to death</td>
<td>3 months</td>
<td>More than 4 months</td>
<td>5 months</td>
<td>Not stated</td>
<td>More than 16 months</td>
</tr>
<tr>
<td>Latex fixation</td>
<td>Not stated</td>
<td>Not stated</td>
<td>Not stated</td>
<td>Not stated</td>
<td>Weak positive</td>
</tr>
<tr>
<td>L. E. prep.</td>
<td>Not stated</td>
<td>Not stated</td>
<td>Not stated</td>
<td>Not stated</td>
<td>Early: negative; later: strongly positive</td>
</tr>
<tr>
<td>Marrow (myeloid) chromosomes</td>
<td>PH⁺-absent; C-trisomy; appreciable ploidy of high degree</td>
<td>PH⁺-absent; C-trisomy; normal diploid</td>
<td>PH⁺-absent; C-trisomy; normal diploid; no polyploidy</td>
<td>C-trisomy + normal diploid; no polyploidy</td>
<td>C-trisomy; no polyploidy</td>
</tr>
<tr>
<td>Peripheral blood chromosomes (lymphocyte; plus myeloid in some cases)</td>
<td>Late: normal diploid; Early: C-trisomy and diploid</td>
<td>Not stated</td>
<td>C-trisomy + normal diploid in 48-hr (early) cultures</td>
<td>Normal diploid</td>
<td></td>
</tr>
</tbody>
</table>

a. Corrected from Winkelstein et al. (1).

reports and other data to either prove or disprove the contention.

REFERENCES
4. K. H. Rothfels and L. Siminovitch, An


Iron Absorption After Portocaval Shunt Operations

Milan J. Chernelch, H. Saul Winchell, Thornton W. Sargent, Myron Pollycove and Carol J. Shkurkin

SUMMARY

Intestinal iron absorption was studied in 15 cirrhotic patients, 7 of whom had had previous end-to-side portocaval anastomosis. In general intestinal iron absorption was subnormal for both groups studied. No evidence of increased intestinal iron absorption secondary to surgical portocaval shunting was obtained from these studies in cirrhotic patients. In two patients studied both before and after the operation intestinal absorption of iron diminished following the shunting procedure. In four normal dogs intestinal iron absorption was found to increase subsequent to portocaval anastomosis. It is suggested that end-to-side portocaval shunting of blood in the dogs in this series resulted in increased portal venous pressure, whereas the similar procedure in cirrhotic patients resulted in decreased portal venous pressure. The alterations in intestinal absorption noted in the recent experiments may be related to such alterations in portal venous pressure. Our results in patients following portocaval shunting suggest that the previously reported hepatic siderosis in these patients is due to factors other than increased intestinal iron absorption.

Previous reports suggest that iron metabolism may be altered after surgical portocaval shunting in patients with hepatic cirrhosis.

Enhanced deposition of iron in the liver has been reported associated with cell damage and fibrosis (1-7).

Different etiologic associations, among them folic acid or pyridoxine deficiency, prolonged iron ingestion, or excessive amounts of iron administered by blood transfusions, have been implicated in the pathogenesis of these findings. The purpose of this work was to determine whether an increase in iron absorption could contribute to this reported increase of hepatic iron following this operation.

MATERIALS AND METHODS

Oral iron absorption was studied in 15 cirrhotic patients, 7 of whom had prior end-to-side anastomosis of the portal vein to the inferior caval vein for treatment of the consequences of portal hypertension. Two patients were studied both before and after the performance of this operation. In these seven patients the interval between the portocaval shunting procedure and the performance of the oral iron absorption study varied from 5 weeks to 5.5 years. Four dogs were studied prior to and at 6 weeks and 4 months following similar end-to-side anastomosis of the portal vein to the inferior vena cava.

Intestinal iron absorption was evaluated according to the following protocol. Subsequent
Fig. 1. Relationship between percent absorption in iron (4 mg carrier dose) and percent circulating reticulocytes in human subjects before and after portocaval shunt operation. The numbers adjacent to each point represent the serum iron values in μg. Previously determined normal range is shown as the shaded area (8).

Table I. Hematologic values and results of iron absorption studies in patients with cirrhosis with and without surgical portocaval shunts. The patients whose initials are underlined were studied both prior to and subsequent to surgical portocaval shunting.

<table>
<thead>
<tr>
<th>Time after operation</th>
<th>Patient's initials</th>
<th>Hgb (g%)</th>
<th>Serum iron (μg%)</th>
<th>Reticulocyte (%)</th>
<th>Oral Fe absorption (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cirrhotic patients without portocaval shunts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R. R.</td>
<td>-</td>
<td>33</td>
<td>1.8</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>S. W.</td>
<td>-</td>
<td>77.5</td>
<td>1.5</td>
<td>5.3</td>
<td></td>
</tr>
<tr>
<td>T. A.</td>
<td>-</td>
<td>51</td>
<td>0.8</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>H. J.</td>
<td>-</td>
<td>60</td>
<td>4.0</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>P. D.</td>
<td>-</td>
<td>55</td>
<td>3.4</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>E. D.</td>
<td>-</td>
<td>53.5</td>
<td>4.1</td>
<td>55.8</td>
<td></td>
</tr>
<tr>
<td>K. K.</td>
<td>9.2</td>
<td>-</td>
<td>3.2</td>
<td>9.5</td>
<td></td>
</tr>
<tr>
<td>G. R.</td>
<td>10.2</td>
<td>98</td>
<td>7.2</td>
<td>18.3</td>
<td></td>
</tr>
<tr>
<td><strong>Cirrhotic patients with portocaval shunts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 w</td>
<td>B. L.</td>
<td>-</td>
<td>92.5</td>
<td>1.4</td>
<td>56.5</td>
</tr>
<tr>
<td>5.5 y</td>
<td>M. A.</td>
<td>14.1</td>
<td>108</td>
<td>2.2</td>
<td>1.8</td>
</tr>
<tr>
<td>5 y</td>
<td>V. J.</td>
<td>12.0</td>
<td>202</td>
<td>1.2</td>
<td>4.2</td>
</tr>
<tr>
<td>6 w</td>
<td>G. F.</td>
<td>11.6</td>
<td>40</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>4 y</td>
<td>C. M.</td>
<td>13.0</td>
<td>43</td>
<td>1.4</td>
<td>3.0</td>
</tr>
<tr>
<td>5 w</td>
<td>E. D.</td>
<td>13.6</td>
<td>-</td>
<td>2.0</td>
<td>4.1</td>
</tr>
<tr>
<td>6 w</td>
<td>K. K.</td>
<td>12.2</td>
<td>152</td>
<td>0.9</td>
<td>4.3</td>
</tr>
</tbody>
</table>
Fig. 2. Iron absorption in dogs before, 6 weeks after, and 4 months after portocaval shunting. The numbers along the abscissa corresponding to each bar are the dog identification numbers. Percent of the administered dose absorbed in 2 weeks is plotted on the ordinate. The mean value (X) is shown for each group and is connected by the solid line.

Hematologic values were determined in the routine fashion, and determinations of serum iron and latent iron binding capacity were performed according to the technique of Peters et al. (9, 10).

Table II. Hematologic values and results of iron absorption studies in dogs prior to and at 6 weeks and 4 months following surgical portocaval shunting.

<table>
<thead>
<tr>
<th>Dog No.</th>
<th>Hct. (0/0)</th>
<th>Retic. (%)</th>
<th>Serum iron (μg %)</th>
<th>TIBC (μg %)</th>
<th>Oral iron absorption (%)</th>
<th>Plasma $^{59}$Fe $T_{1/2}$ (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preoperatively</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>136</td>
<td>31.5</td>
<td>0.1</td>
<td>215</td>
<td>397</td>
<td>4.5</td>
<td>1.08</td>
</tr>
<tr>
<td>137</td>
<td>35</td>
<td>0.1</td>
<td>120</td>
<td>354</td>
<td>9.2</td>
<td>1.47</td>
</tr>
<tr>
<td>139</td>
<td>44</td>
<td>0.2</td>
<td>140</td>
<td>392</td>
<td>10.4</td>
<td>1.1</td>
</tr>
<tr>
<td>117</td>
<td>40.5</td>
<td>0.1</td>
<td>119</td>
<td>458</td>
<td>5.8</td>
<td>-</td>
</tr>
<tr>
<td>One month after shunt</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>136</td>
<td>36</td>
<td>0.3</td>
<td>105</td>
<td>403</td>
<td>10.1</td>
<td>0.40</td>
</tr>
<tr>
<td>137</td>
<td>42</td>
<td>0.7</td>
<td>71</td>
<td>345</td>
<td>11.2</td>
<td>0.55</td>
</tr>
<tr>
<td>139</td>
<td>38.8</td>
<td>0.4</td>
<td>108</td>
<td>346</td>
<td>18.2</td>
<td>0.45</td>
</tr>
<tr>
<td>Four months after shunt</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>136</td>
<td>33</td>
<td>0.2</td>
<td>136</td>
<td></td>
<td>17.1</td>
<td>0.95</td>
</tr>
<tr>
<td>137</td>
<td>34.75</td>
<td>0.1</td>
<td>114</td>
<td></td>
<td>18.8</td>
<td>1.35</td>
</tr>
<tr>
<td>139</td>
<td>34.5</td>
<td>0.1</td>
<td>138</td>
<td></td>
<td>19.5</td>
<td>1.6</td>
</tr>
<tr>
<td>117</td>
<td>28</td>
<td>0.3</td>
<td>160</td>
<td></td>
<td>9.9</td>
<td>1.45</td>
</tr>
</tbody>
</table>
RESULTS

Table 1 and Fig. 1 summarize the results of iron-absorption studies on cirrhotic human subjects with and without prior surgical portocaval shunting procedures. Table 2 and Fig. 2 summarize the results obtained on four dogs prior to and at 1 and 4 months subsequent to end-to-side portocaval shunting. The normal limits of the relationship between percent absorption of a 4-mg oral iron dose and the percent circulating reticulocytes are presented in Fig. 1 as the shaded area (8). It can be appreciated that most patients with cirrhosis (in the presence or absence of surgical portocaval shunts) have a lower percent absorption of an oral iron dose than that shown for the "normal range." In the two patients studied both prior to and subsequent to the portocaval shunting procedure a diminution in the percent of the absorbed oral iron dose was found subsequent to the operation. In general those patients with cirrhosis who had greater than "normal" iron absorption also had concomitant low serum iron concentrations.

It can be seen from Fig. 2 that iron absorption increased in dogs subsequent to end-to-side portocaval anastomosis. Furthermore in these dogs iron absorption continued to increase during the 6-week to 4-month interval following portocaval shunting. The increased oral iron absorption after 4 months is particularly noteworthy in that at this time serum iron concentrations and the plasma radioiron clearance rate had returned to approximately normal preoperative levels. At 4 months following the portocaval shunting procedure two of the animals had hematocrit values comparable to the preoperative levels, whereas two of them had diminished venous hematocrit levels. All, however, showed an increase in oral iron absorption above their base-line values.

DISCUSSION

Rubin and co-workers (11) found hepatic siderosis in rats following end-to-side portocaval anastomosis and concluded that shunting of portal blood results in hepatic siderosis even in the absence of cirrhosis. Our results in dogs in this communication are consistent with those reported by Rubin. It is noteworthy that similar results were generally not obtained in our human patients in that there did not appear to be a consistent increase in intestinal iron absorption following portocaval shunts in cirrhotic patients with portal hypertension. The apparently inconsistent results obtained in dogs and man in this paper might be explained by an observation by one of the authors at the time of the portocaval shunting operation in the dogs: End-to-side portocaval anastomosis in the dogs reported in this paper appeared to result in partial restrictions of portal venous flow such that portal venous pressure was greater following the operation than prior to the operation. It is suggested that portocaval shunting procedures in normal dogs (at least in the hands of the present operator) resulted in increased portal venous pressure with subsequent increased blood pooling in the venous end of the capillaries of the gut. It is possible that intestinal iron absorption may be somehow related to the blood pool size or the venous pressure within the gut. If this were the case then one could explain our present results by postulating that surgical portocaval shunting in the normal dog resulted in increased portal venous pressure, whereas portocaval shunting in the person with cirrhosis resulted in decreased portal venous pressure, the former acting to increase intestinal iron absorption and the latter acting to decrease intestinal iron absorption.

The inability to find consistently increased iron absorption in patients subsequent to porto-
caval shunting suggests that the hepatic siderosis previously described may be related to factors other than increased intestinal iron absorption. It is also possible that following surgical portocaval anastomosis only certain patients develop markedly increased iron absorption (note patient B. L. in this report who, after the operation, had 56% absorption of the oral dose). One wonders whether in such cases the portal venous pressure was actually increased following the operation, as could occur if there were thrombotic occlusion of the anastomotic site.

REFERENCES

Increased Iron Absorption in Hemochromatosis
Before and After Phlebotomy Therapy

Thornton W. Sargent, Hiroshi Saito and H. Saul Winchell

SUMMARY

Idiopathic hemochromatosis has been considered to be a heritable disease in which excessive iron is absorbed from the diet, eventually leading to organ damage from the toxic accumulation of iron. Lack of reliable iron absorption measurements in such patients has led to an alternative hypothesis of the etiology of the disease. In the work described here, utilizing a whole-body counter, a correlation has been shown in normal subjects between oral iron absorption and circulating reticulocytes; patients with plasma iron levels above and below normal have iron absorption below and above, respectively, the confidence limits of the regression line of the correlation. Eight patients with idiopathic hemochromatosis were given iron absorption tests before, during, and after phlebotomy therapy. In all cases the study after therapy was at a time when the plasma iron levels were within the normal range and all blood indices were normal, and in every case at this time the iron absorption was above the normal range. The absorption of iron, compared with the body need for it, was considered to be in excess of need before therapy as well. These results are considered to support the concept that in patients with hemochromatosis there is a congenital failure of control of iron absorption, leading to excessive accumulation of this element in the body.

Endogenous hemochromatosis has been considered to be a hereditary defect leading to increased absorption of iron from the diet with subsequent gradual accumulation of iron in liver and other organs, the various symptoms and signs of the disease apparently resulting from tissue damage due to excessive iron deposition (1, 2). An alternative hypothesis proposed by McDonald (3) is that the disease is a form of cirrhosis complicated by excessive oral iron intake, rather than an abnormal increase in absorption of iron from a normal diet. Evidence for abnormally high iron absorption in patients with this disease would weigh against this latter hypothesis.

Williams et al. (4) reported results of iron-absorption studies in a series of hemochromatotic patients before, during, and after venesection therapy. Although in no case was a single patient studied in all three stages, the four patients studied when all blood indices had returned to normal showed markedly elevated iron absorption, as did most of the patients studied during venesection therapy. Patients before therapy had absorption within the normal range, but because of their elevated iron stores such normal absorption was considered by the authors to be in excess of need.
In this communication we describe serial iron absorption studies in eight patients with endogenous hemochromatosis, performed before, during, and after phlebotomy therapy, and relate the results to the state of erythropoiesis and iron stores at the time of each study.

MATERIALS AND METHODS

Eight hemochromatotic patients were examined in this study. The diagnosis was suggested by combinations of the clinical features of diabetes, skin darkening, loss of axillary hair, loss of potency, hepatomegaly, abnormally elevated serum-iron concentration (> 170 μg percent), and the characteristic markedly increased deposition of iron shown on hepatic biopsy. In all cases the diagnosis was confirmed by the patient’s tolerance to phlebotomy in excess of 25 liters prior to iron depletion. In most cases skin biopsy revealed iron deposition around hair and sweat follicles. Five of the eight cases had neither alcoholism or previous hepatitis in their histories. Ferrokinetic studies performed on each propositus according to the method of Pollycove and Mortimer (5) demonstrated abnormally rapid early deposition of iron in the liver. Net plasma-iron clearance was increased and the incorporation of radioiron into red cells was decreased below normal levels, typical of hemochromatosis (5).

The iron absorption tests were performed as described by Saito et al. (6). An oral dose of 1 to 4 μCi of 59Fe with 4 mg ferrous sulfate carrier was administered to the fasting subject, and food was withheld for an additional 2 hr. The subject returned 2 weeks after the oral administration of the test and was counted in the whole-body counter. The radioiron in the body at this time is a measure of total body iron absorption, in contrast to iron absorption measured by the double-isotope technique. The latter method measures only that iron absorbed from the intestinal tract which reaches the systemic plasma. When the plasma iron concentration is elevated, some of the absorbed iron is deposited in the liver as a first-pass phenomenon and does not reach the systemic plasma (7). Thus absorption measured by the double-isotope technique in hemochromatotic patients with elevated plasma iron may be in error.

Hemoglobin concentration and hematocrit were determined in the routine manner. Reticulocyte counts were performed using slide incubation with new methylene blue. A total of 1000 red blood cells were observed for reticulum content. Serum iron concentration was determined by the modification by Peters and co-workers of the Ramsey method (8) (normal 70 to 170 μg %). Total iron binding capacity was performed by the method of Peters et al. (9) (normal 200 to 400 μg %).

The treatment schedule in the patients with hemochromatosis consisted of one to two 500-ml phlebotomies per week as tolerated. Depletion of iron stores was considered to be complete when the patient no longer tolerated phlebotomy without a precipitous drop in circulating Hgb concentration, when circulating red blood cells were markedly microcytic and hypochromic, serum iron concentration was less than 70 μg % for at least 1 month after the last phlebotomy, and the bone marrow aspirate showed little or no stainable iron. Repeat liver biopsies were generally not performed to directly evaluate hepatic iron stores.

Normal subjects were studied in a similar manner. In three normal subjects the effect of increased erythropoiesis was studied by performing the absorption test before and 5 days after a 500-ml phlebotomy. The effect of plasma iron concentration was studied by
performing the absorption test in iron-deficient patients while their serum iron concentration was low, and again in two such patients when their plasma iron concentration was elevated following intravenous administration of iron dextran (Imferon) 1 to 2 weeks prior to the study.

RESULTS

Relationship between oral iron absorption, reticulocyte count, and plasma iron concentration in nonhemochromatotic subjects

The relationship between the percent absorption of a test oral iron dose as a function of the percent reticulocytes in peripheral blood of normal subjects is shown in Fig. 1a. This correlation has been reported previously (6), but is shown here with 16 additional subjects. The correlation coefficient is 0.77; the 95% confidence limits for prediction of further measurements are represented by the shaded area, and since they are based upon observed data the limits include random errors of both absorption and reticulocyte measurements. The correlation of absorbed dose with reticulocyte count is in keeping with the concept that iron absorption is proportionate to the amount needed for red cell production. It might be expected that oral iron absorption would correlate equally well with plasma iron turnover rate. However, plasma iron turnover varies widely on a diurnal basis, and further, would be altered by the administration of the test dose itself. Since reticulocytes are recognizable as young cells for a period of about 2 days, their relative abundance is an index of erythropoiesis averaged over this time.

The correlation with reticulocyte count was further tested by giving the absorption test to three subjects before and five days after a 500-ml phlebotomy. As seen in
Fig. 2. Iron absorption in patients with iron-deficiency anemia. The numbers inside each point indicate the serum iron concentration at the time the test dose was given.

DBL 703-5595

Fig. 1b, the reticulocyte count increased following phlebotomy, as expected, with a proportional increase in iron absorption, the latter remaining in the normal range as defined above. Serum-iron concentration remained within the normal range prior to and subsequent to phlebotomy in these subjects.

In patients with iron-deficiency anemia, in whom the plasma iron concentration is below the normal values (< 70 μg %), iron absorption as a function of reticulocyte count is above the normal range, as shown in Fig. 2. In two such patients who had intravenous dextran-bound iron (Imferon) injections 1 to 2 weeks previous to the test, iron absorption was depressed to well below the normal range; the plasma iron concentrations were abnormally elevated during this time interval but the patients were still anemic and undoubtedly continued to have markedly increased erythropoiesis. The results on these normal, iron-deficient, and acutely iron-loaded patients may be summarized as follows:

1. In normal subjects the fraction of iron absorbed from a normally administered test dose varies directly with the reticulocyte count.

2. In iron-deficiency anemia absorption of iron is increased disproportionate to the reticulocyte count. When two such patients were given sufficient iron intravenously to abnormally elevate the serum iron concentration, iron absorption was decreased disproportionate to the reticulocyte count despite only a mild change in the degree of anemia.

Iron absorption in patients with hemochromatosis

The eight patients with endogenous hemochromatosis were given iron absorption tests at three stages of their treatment: (a) at the time of presentation, when there had been either no previous phlebotomy or at most only a few; (b) in some cases during the course of phlebotomy therapy, while the serum iron was still elevated; (c) after the completion of phlebotomy therapy, in some cases when the patient was still iron-deficient as indicated by lowered hemoglobin concentration and serum iron concentration, and in every case at least 2 months after the last previous phlebotomy, at a time when the patient was normochromic and normocytic, and had a serum iron within the normal range (70 to 170 μg %). Without further phlebotomy certain patients experienced a rather abrupt increase in serum iron concentration above the normal range, and repeat absorption tests were performed at that time.
Table I. Clinical data, blood indices, and results of iron absorption tests on eight patients with hemochromatosis before, during, and after phlebotomy therapy.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age when studied</th>
<th>Alcoholism (+ or 0)</th>
<th>Diabetes (+ or 0)</th>
<th>Days since last phlebotomy</th>
<th>Litters blood removed prior to time of study</th>
<th>Hemoglobin (g/100 ml)</th>
<th>Serum iron conc. (mg%)</th>
<th>Total serum iron binding capacity (mg %)</th>
<th>Saturation of plasma transferrin (%)</th>
<th>Circulating reticulocytes</th>
<th>Percent of 4 mg Fe ++ absorbed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF M</td>
<td>50</td>
<td>0</td>
<td>+</td>
<td>180</td>
<td>58</td>
<td>14.8</td>
<td>14.9</td>
<td>72</td>
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Table I summarizes the results on eight hemochromatotic patients, with pertinent clinical information. In Fig. 3, a-h, each figure shows the results of the series of absorption tests on a single patient, the direction of the arrows in-
Fig. 3. (a–h) Iron absorption of eight hemochromatotic patients before, during, and after phlebotomy therapy. SI at time of each test is shown beside the data point. The arrows indicate the chronological sequences of the tests.

indicating the chronological sequence, and the number beside the point for each test giving the serum iron concentration at that time.

Patient A. F. (Fig. 3a) had greatly suppressed absorption before phlebotomy. Six months after completion of a 58-liter course of phlebotomies which produced a hypochromic microcytic anemia, depleted bone marrow iron stores, and subnormal serum iron concentration, two repeat iron-absorption studies showed that absorption was greatly elevated even though the serum iron had spontaneously risen again to abnormally high levels (226 and 257 μg % respectively). Only an additional 5 liters of blood was removed before the patient developed all the signs of iron depletion seen after the first course of phlebotomy. Four months after the second course of phlebotomy, when his serum iron concentration was 72 μg %, a repeat iron-absorption study again was abnormally high.

Patient E. B. (Fig. 3b) when tested initially had high absorption (40%) despite an elevated serum iron concentration of 176 μg %. In relationship to reticulocytosis this was considered to be within the normal range. Subse-
quent to removal of 55 liters of blood two further absorption tests at 42 and 240 days after the last phlebotomy showed absorption well above the normal range at a time when the serum iron concentration was within normal limits.

Patient J. R. (Fig. 3c) was first studied after removal of 21 liters of blood, 49 days after the last phlebotomy, and the absorption was found to be within the normal range, despite a serum iron concentration of 250 μg %. After depletion of his iron stores, at a time when he was quite iron-deficient (serum iron 3 μg %), his absorption was elevated as would be expected from iron deficiency alone. Two years later, with occasional maintenance phlebotomies, his SI was within normal limits (149 μg %) but absorption was still elevated. In the succeeding 2 months, his SI rose spontaneously to 193 μg %, at which time absorption was suppressed almost to within the normal range.

Patient T. G. (Fig. 3d) was first studied after phlebotomies had been initiated; at that time his serum iron concentration was still elevated, and his absorption was within the normal range. After depletion of stores and while still iron-deficient (serum iron concentration 41 and 39 μg %), two absorption tests 77 and 240 days after the last phlebotomy showed the elevated absorption characteristic of iron deficiency. However, a year later, the serum iron concentration had risen spontaneously to a normal level (106 μg %), and absorption was still elevated. Without further phlebotomy, the serum iron concentration rose to above-normal levels (173 μg %) and absorption at this time was suppressed to within the normal range.

Patient M. T. (Fig. 3e) had had no phlebotomies when first studied (her serum iron concentration at that time was 219 μg %) and absorption was within the normal range. The next test was performed 63 days after completion of a 31-liter course of phlebotomy, when the patient had abnormally low serum iron concentration (47 and 66 μg %) and absorption was above normal. By 123 days after completion of phlebotomy the SI had risen almost to the normal range (68 μg %) but absorption was still elevated. The serum iron then rose to 216 μg % and absorption fell to almost within the normal range. Subsequently without further phlebotomy the serum iron concentration fell to within the normal range (150 μg %) and absorption was again found to be elevated.

Patient J. T. (Fig. 3f), when first studied before any phlebotomies, had absorption just within the normal range. One hundred and sixty-five days after the removal of 50 liters of blood had depleted iron stores and the patient was iron-deficient, repeat study showed that the serum iron had already risen to 206 μg % and absorption was within the normal range. After further phlebotomies this sequence was essentially repeated, the SI rising almost above normal (162 μg %) and the absorption still within normal range. After removal of an additional 5.5 liters of blood, an absorption test 62 days later showed an above-normal absorption with the SI an almost-normal 62 μg %.

Patient R. G. (Fig. 3g) was the only patient studied who showed an elevated absorption when first studied (serum iron concentration 204 μg %); a subsequent test, near the end of his phlebotomy regime but with his SI still elevated (234 μg %), gave a similar result. Following depletion of iron stores after removal of 51 liters of blood, his serum iron was found to be 100 μg % 130 days after completion of phlebotomy and his absorption remained abnormally elevated.
Patient P. G. (Fig. 3h) was first studied before any phlebotomy therapy; her absorption was within the normal range with SI 281 μg %. Following a phlebotomy regimen in which 26.8 liters of blood was removed, an absorption test 104 days after the last phlebotomy was above the normal range with SI 273 μg %. Removal of an additional 3 liters of blood depleted the patient's iron stores and 240 days later, with SI 29 μg %, iron absorption was elevated as in iron deficiency. After maintenance phlebotomies totaling 2 liters, 90 days after the last one the serum iron was a normal 132 μg % and absorption was still elevated.

The results on these eight patients may be summarized as follows:

(a) When the plasma iron concentration was elevated and the absorption test was given prior to phlebotomy (or after prior phlebotomies in two cases), iron absorption was within the normal range with regard to reticulocyte count in six out of eight cases; in the other two, one case was above and the other below this range.

(b) When iron stores had been depleted and the plasma iron concentration was below normal, but in the absence of significant anemia, iron absorption was always above the normal range, as occurs in nonhemochromatotic patients with iron deficiency.

(c) When depleted patients were tested 2 months or more after their last phlebotomy, at a time when they were normochromic and normocytic and the plasma iron concentration was within normal limits, the iron absorption with regard to reticulocyte count was above the normal range in eight out of eight patients.

Parameters that affect intestinal absorption of iron have been studied by many workers, and much of this has recently been summarized (10). Some years ago it was shown that an increased level of erythropoiesis generally increased absorption of iron and that iron loading tended to decrease it (11), yet accurately measurable correlations between absorption and either erythropoiesis or iron loading could not be obtained (12). In the study reported here it was found that in normal subjects the fraction of iron absorbed varies directly with circulating reticulocytes, supporting the concept that it is directly related to erythropoietic rate. Failure of other workers to observe this relationship may have been due to use of measures of absorption less accurate than a whole-body counter and use of doses of carrier iron, in many cases, that were far above or below normal levels. Wide variations in absorption among normal subjects and in a single subject at different times have been found by many workers (10). With the method used here, similar variations occur, but always within the confidence limits of the regression line shown in Fig. 1a. The confidence limits of this regression thus define a narrower range of normal absorption than was previously possible.

When the plasma iron falls below normal as in patients with iron deficiency anemia, absorption is above the normal range as defined above. In two such patients, when the plasma iron had been abnormally elevated by intravenous iron-dextran, oral iron absorption was suppressed below the normal range. Sölvell (13) also found an inverse relationship between absorption and plasma iron, but he utilized acutely elevated plasma iron. Wheby and Jones (14), however, showed that when iron is absorbed in the presence of saturated transferrin (in rats), it is deposited in the liver on its first pass through the portal system; this phenomenon was ultimately demonstrated in humans (7). Wheby and Jones did not find that acutely elevated plasma iron reduced absorption, and attributed Sölvell's results to the first-pass phenomenon. In the two patients reported here the
Iron absorption in hemochromatosis related to phlebotomy

Saturation of transferrin was chronic rather than acute, which more closely simulates the situation in hemochromatosis, yet is complicated by the presence of the nonnatural dextran-bound iron. The suppressed absorption in these two patients could be interpreted as support for the modified mucosal block theory of Crosby (15), in that the chronically high levels of plasma iron would have time to saturate the developing mucosal cells and suppress absorption by the mechanism he has proposed. On the other hand it is hard to see how such a mechanism could account for control of absorption according to the erythropoietic level. The 3 days required for elaboration of the intestinal mucosal cells before they can influence iron absorption (according to Crosby) is also approximately the period required for erythropoietic marrow to respond to an increased need for red cells (11). Thus there is still no clear evidence on which to distinguish between a mucosal and an erythropoietic control mechanism; indeed, perhaps the mucosal mechanism acts upon information from the erythropoietic marrow, although no mechanism for such information transfer has been found.

Untreated patients with hemochromatosis generally have elevated plasma iron concentrations, and one would thus expect iron absorption to be suppressed. The fact that absorption in these patients was found to be usually in the normal range indicates that absorption is nevertheless relatively increased. Furthermore, after depletion of iron stores and at a time when plasma iron concentration and other blood indices were normal, iron absorption was markedly above the normal range. Thus in hemochromatotic patients it appears that at all times oral iron absorption is in excess of need.

The measurement of oral iron absorption in hemochromatotics may be of value in assessing rate of repletion of iron stores, which is not possible by other methods such as plasma iron concentration or liver biopsy. It is to be noted that some patients judged to be iron-depleted were found to have elevated plasma iron concentrations within a year following cessation of phlebotomy. It is possible that these patients had very slowly equilibrating stores of iron even when they were judged to be depleted, or that hemochromatotics can have elevated plasma iron when iron stores are not excessive.

Oral iron absorption as described here may be useful in defining the physiologic basis and mode of transmission of the disease, and in detecting it in family members early enough to begin prophylactic phlebotomy and thus prevent irreversible damage. The results reported here support the view that hemochromatosis has, as a basic predisposing factor, a congenital defect which permits excessive absorption of iron from a diet containing normal amounts of this element.

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Effects of Large Doses of Unlabeled L-Methionine on 14CO2 Production in Rats Given L-Histidine (imidazole-2-14C), L-Serine-3-14C, Formimino-14C L-Glutamic Acid, and 14C-Formate

Tran Manh Ngo and H. Saul Winchell

SUMMARY Following administration of L-histidine (imidazole-2-14C), L-serine-3-14C, and formimino-14C L-glutamic acid to normal rats that had been given large doses of L-methionine, the initial rate and amount of 14CO2 excreted in the breath are significantly decreased, whereas following administration of 14C-formate, 14CO2 appearance is unchanged in methionine-treated rats. These results indicate the presence of in vivo competition for physical transport of these amino acids across cell membranes.

In previous studies, competition between several amino acids for intestinal absorption has been proposed (2-5). This competition was not found in kidney tissue (6). Methionine was found to cause a large inhibition in the uptake of many amino acids in brain slices of rats (7). In folic-acid- and vitamin-B12-deficient rats given massive doses of L-methionine, the amount of 14CO2 production from the #2 carbon of the imidazole ring of histidine was shown to be significantly increased (1).

In the study presented here, we demonstrate significant alterations in the catabolism of L-histidine, L-serine, and formimino glutamic acid in normal rats given loading doses of L-methionine, by measurements of 14CO2 production in methionine-treated rats subsequent to the intravenous administration of L-histidine (imidazole-2-14C), L-serine-3-14C, and formimino-14C L-glutamic acid.

PREPARATION OF EXPERIMENTAL ANIMALS

Inbred male Buffalo rats (Simonsen Laboratory, Gilroy, California) weighing 240 to 245 g were used in all experiments.

L-histidine (imidazole-2-14C)

In the first series of studies, 35 rats were divided into two groups: 13 methionine-treated rats and 22 control rats and rats given no treatment prior to the administration of the 14C-labeled histidine. Methionine-treated rats were subdivided further into two subgroup received intravenously 40 mg of L-methionine (General Biochemicals, Laboratory Park, Chagrin Falls, Ohio) mixed homogeneously with 2.5 μCi of L-histidine imidazole-2-14C (specific activity: 267 μCi/mg; Nuclear Chicago Corp., Des Plaines, Ill.). In the second subgroup 40 mg of L-methionine was given to three rats each at 10, 40, and 120 min prior to the
intravenous injection of 2.5 μCi of L-histidine (imidazole-2-^{14}C).

In each study, each rat received 2.5 μCi of L-histidine (imidazole-2-^{14}C) intravenously under light anesthesia with diethyl ether, and the appearance of \(^{14}\text{CO}_2\) in the breath was determined.

Formimino-^{14}C L-glutamic acid

In the second series of studies, eight male Buffalo rats were divided into two groups of four control rats and four methionine-treated rats. The experimental rats received intravenously 40 mg of L-methionine 10 and 40 min (two rats each) prior to the intravenous administration of 10 μCi of ^{14}C-formimino L-glutamic acid (specific activity: 1.0 mCi/10.1 mg; New England Nuclear Corp., 575 Albany Street, Boston, Massachusetts 02118).

L-serine-3-^{14}C

In the third series of studies, 12 male Buffalo rats were divided into two groups of six control and six methionine-treated rats. Experimental rats were given intravenously 40 mg of L-methionine 10 and 40 min (three rats each) prior to the intravenous administration of 2.5 μCi of L-serine-3-^{14}C (specific activity: 8.5 mCi/mM; Nuclear Chicago, 333 Howard Avenue, Des Plaines, Ill. 60018).

\(^{14}\text{C} \text{formate}\)

In the fourth series of studies, six male Buffalo rats were divided into two groups of three control rats and three methionine-treated rats. Each experimental rat was given intravenously 40 mg of L-methionine 10 min prior to intravenous injection of 0.5 μCi of \(^{14}\text{C} \text{formate}\) (specific activity: 1.00 mCi/14.5 mg; New England Nuclear Corp.).

RESULTS

L-histidine (imidazole-2-^{14}C)

Figure 1 presents curves of composite data expressing the rate of \(^{14}\text{CO}_2\) appearance in the breath of control rats and rats either given a homogeneous mixture of L-methionine and L-histidine (imidazole-2-^{14}C) or given L-methionine prior to the intravenous injection of L-histidine (imidazole-2-^{14}C). The ordinate represents \(^{14}\text{CO}_2\) excretion rate expressed as mCi per min on a logarithmic scale and the abscissa as time in min on a linear scale. Each point represents the mean of the \(^{14}\text{CO}_2\) excretion rate for each group of animals at the
given time, and the length of the vertical bar through each point represents 1 standard error of the mean for each group.

It is clear that there was significantly decreased $^{14}$CO$_2$ production in the rats treated with methionine 10 and 40 min prior to the intravenous injection of the $^{14}$C-labeled histidine. In rats given 40 mg of L-methionine 120 min prior to administration of $^{14}$C-labeled histidine, however, the cumulative excretion of $^{14}$CO$_2$ during the initial 90 min returned to the normal range. No significant difference was noted between $^{14}$CO$_2$ breath curves of control rats and those given a homogeneous mixture of methionine and L-histidine (imidazole-2-$^{14}$C).

**Formimino-$^{14}$C L-glutamic acid**

Figure 2 presents composite data of $^{14}$CO$_2$ breath curves of control and methionine-treated rats. There is a slight difference between $^{14}$CO$_2$ curves of control rats and those of methionine-treated rats.

**L-serine-3-$^{14}$C**

Figure 3 presents the composite data depicting the rate of $^{14}$CO$_2$ excretion in the breath of six control rats and six rats given 40 mg of L-methionine 10 and 40 min prior to the intravenous administration of L-serine-3-$^{14}$C. The appearance of $^{14}$CO$_2$ in the breath of methionine-treated rats was significantly decreased when the treatment was 10 min prior to the intravenous administration of L-serine-3-$^{14}$C, but returned to the normal range when treatment was 40 min prior to the injection of the $^{14}$C-labeled serine.

**$^{14}$C-formate**

Figure 4 presents composite data of $^{14}$CO$_2$ breath curves of control rats and methionine-treated rats. There is no significant difference between $^{14}$CO$_2$ curves of control rats and those of methionine-treated rats.

**DISCUSSION**

The studies presented here demonstrate that large doses of L-methionine decrease the amount of oxidation of the #2 carbon of the imidazole ring of histidine, the #3 carbon of serine, and probably the carbon atom of the formimino glutamic acid to CO$_2$ in vivo, whereas loading doses of L-methionine do not influence the oxidation of $^{14}$C-formate. These results suggest that L-methionine may inhibit the physical transport of L-histidine.
Fig. 3. Composite data of the rate of $^{14}$CO$_2$ appearance in the breath following iv administration of L-serine-3-$^{14}$C preceded by iv injection of 40 mg of L-methionine 10 and 40 min before.

The ordinate represents percent of administered $^{14}$C excreted as $^{14}$CO$_2$ per min and the abscissa represents time in min following iv injection of the $^{14}$C-labeled serine. Each point represents the mean of the $^{14}$CO$_2$ excretion rate for each group of animals at the given time, and the length of the vertical bar through each point represents 1 standard error of the mean.

DBL 703-5603

Fig. 4. Composite data of the rate of $^{14}$CO$_2$ appearance in the breath following iv administration of $^{14}$C-formate preceded by iv injection of 40 mg of L-methionine 10 min before.

The ordinate represents percent of administered $^{14}$C excreted as $^{14}$CO$_2$ per min and the abscissa represents time in min following iv injection of the $^{14}$C-formate. Each point represents the mean of the $^{14}$CO$_2$ excretion rate for each group of animals at the given time, and the length of the vertical bar through each point represents 1 standard error of the mean.

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and L-serine across the cell membrane. The alterations in formimino glutamic acid may be due to inhibition either of physical transport of this amino acid or of biochemical processes involved in the oxidation of monocarbon fragments attached to tetrahydrofolic acid. In contrast, L-methionine in loading doses has no effect on formate. Our data are consistent with the previous findings, which demonstrated that L-methionine decreased uptakes of several amino acids for Ehrlich ascites tumor cells (2, 6), for rat brain slices (7), and for intestinal absorption in normal hamsters (3) and rats (4). Moreover, our results are strongly supported by recent data obtained by Lin and Winchell (8) in this Laboratory confirming that L-methionine apparently depresses the intracellular uptakes of L-histidine and L-serine in equilibrium state. They utilized dog bone marrow cells incubated in Eagle basal medium in the presence of L-histidine (imidazole-2-$^{14}$C) or L-serine-3-$^{14}$C in the presence and absence of L-methionine. The intracellular uptakes of the above amino acids, which were analyzed according to the compartment theory, demonstrated that the fractional turnover rate of intracellular pool, and the influx constant, fractional efflux constant, and fractional utilization rate constant of those amino acids...
EFFECTS OF DOSES OF L-METHIONINE ON $^{14}$CO$_2$ PRODUCTION

Table 1. $T_{\text{max}}$ and integral $^{14}$C excretion determined from $^{14}$CO$_2$ appearance in breath following iv administration of L-histidine (imidazole-2-$^{14}$C), formimino-$^{14}$C glutamic acid, L-serine-3-$^{14}$C, and $^{14}$C-formate in control and methionine-treated rats (the number of animals in each group is noted in parentheses).

<table>
<thead>
<tr>
<th>Category (and number of rats)</th>
<th>$T_{\text{max}}$ (min ± S. E.)</th>
<th>$^{14}$C Excretion in 90 min (% ± S. E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-histidine (imidazole-2-$^{14}$C) Control rats (22)</td>
<td>21.54±0.57</td>
<td>1.20±0.65</td>
</tr>
<tr>
<td>Rats given simultaneous iv injection of the $^{14}$C-labeled histidine mixed with 40 mg L-methionine (4)</td>
<td>22.75±1.65</td>
<td>1.06±0.11</td>
</tr>
<tr>
<td>Rats 10 min after iv administration of 40 mg L-methionine (3)</td>
<td>21.50±2.18</td>
<td>0.77±0.18</td>
</tr>
<tr>
<td>Rats 40 min after iv administration of 40 mg L-methionine (3)</td>
<td>20.12±0.83</td>
<td>0.86±0.05</td>
</tr>
<tr>
<td>Rats 120 min after iv administration of 40 mg L-methionine (3)</td>
<td>19.00±0.76</td>
<td>1.14±0.16</td>
</tr>
<tr>
<td>Formimino-$^{14}$C L-glutamic acid Control rats (6)</td>
<td>28.25±4.61</td>
<td>0.39±0.07</td>
</tr>
<tr>
<td>Rats 10 min after iv administration of 40 mg L-methionine (2)</td>
<td>37.5</td>
<td>0.31</td>
</tr>
<tr>
<td>Rats 40 min after iv administration of 40 mg L-methionine (2)</td>
<td>34.25</td>
<td>0.38</td>
</tr>
<tr>
<td>L-serine-3-$^{14}$C Control rats (6)</td>
<td>16.83±1.48</td>
<td>9.03±0.72</td>
</tr>
<tr>
<td>Rats 10 min after iv administration of 40 mg L-methionine (3)</td>
<td>16.67±1.04</td>
<td>6.70±0.34</td>
</tr>
<tr>
<td>Rats 40 min after iv administration of 40 mg L-methionine (3)</td>
<td>14.17±0.60</td>
<td>8.65±0.50</td>
</tr>
<tr>
<td>$^{14}$C-formate Control rats (3)</td>
<td>12.67±1.20</td>
<td>7.57±0.27</td>
</tr>
<tr>
<td>Rats 10 min after iv administration of 40 mg L-methionine (3)</td>
<td>13.33±0.17</td>
<td>7.65±0.36</td>
</tr>
</tbody>
</table>

were definitely depressed by large doses of L-methionine. Recent studies by Stahelin and Winchell (9) in this Laboratory showed that administration of large doses of L-methionine to folate-acid-deficient persons caused abnormal mentation in these patients. These induced behavioral changes may be due to a strong inhibition by L-methionine on the uptakes of other amino acids in various tissues, including brain tissue.

The $^{14}$CO$_2$ breath-analysis technique should be useful in future study of competition for metabolism between various materials.

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9. H. Stahelin and H. S. Winchell, Induction of Psychotic Behavior in Folic-Acid-Deficient Patients by Ingestion of L-Methionine, this report.
Free Radicals in Pyrimidines: 
ESR of Irradiated Single Crystals of Orotic Acid

Paul Karl Horan and Wallace C. Snipes

SUMMARY  Hydrogen abstraction from N(1) occurs when single crystals of orotic acid are exposed to ionizing radiation at room temperature. The stable free radical has unpaired spin density on both N(1) and C(5) of the pyrimidine ring. Hyperfine coupling to the nitrogen nucleus shows axial symmetry, with $A_N^N = 15.0$ gauss and $A_N^N = 0$. The hydrogen bonded to C(5) has principal hyperfine values, in gauss, of $A_5^H = 7.3$, $A_5^H = 22.2$, and $A_5^H = 15.1$. From the hyperfine couplings, the spin densities calculated for N(1) and C(5) respectively are 0.29 and 0.65. Principal values of the g-tensor are $g_u = 2.0059$, $g_v = 2.0052$, and $g_w = 2.0023$. The ESR analysis can be used to predict the molecular orientation in orotic acid crystals.

When organic crystalline solids are exposed to ionizing radiation at room temperature, the stable free radicals formed are usually neutral species resulting from the rupture of covalent bonds. In the majority of studies in which positive radical identification has been possible, the mechanism of radical formation involves either hydrogen abstraction from, or hydrogen addition to, the parent molecule. These reactions are usually quite selective, occurring at only one of several possible sites on the molecule. The basis for this selectivity is not fully understood, and no adequate method exists for predicting which stable radical will be formed by irradiating a particular compound.

It appears especially difficult to predict the site of free-radical formation in pyrimidines, in which both hydrogen abstraction and hydrogen addition reactions have been found to occur. It is not too surprising that compounds containing the thymine ring are subject to hydrogen addition reactions at the 5, 6 double bond (1-3), whereas dihydro-thymine undergoes hydrogen abstraction at these same positions (4, 5). However, in cytosine, stable radicals formed both by hydrogen abstraction from N(1) and by hydrogen addition to C(5) of the pyrimidine ring have been identified (6). An interesting hydrogen addition reaction occurs in 5-nitro-6-methyl-uracil, where the hydrogen adds to the keto oxygen at the 4-position (7). The resulting radical has unpaired spin density at both C(4) and C(6), due to resonance with the $\pi$ electrons in the 5, 6 double bond. Hydrogen addition to the oxygen atom bound to C(4) is also thought to occur in halogenated derivatives of uracil (8). Thus a variety of mechanisms for radical formation are seen, and it is not clear what factors determine which mechanism pre-
vails for a particular pyrimidine derivative.

Our work, aimed toward understanding the mechanisms of free-radical formation in irradiated pyrimidines, has now been extended to include a carboxylated derivative of uracil. We report here a detailed ESR study of free radicals in irradiated orotic acid (uracil-6-carboxylic acid). The primary free radical stable at room temperature has been identified and the parameters characterizing its ESR absorption have been determined. It was found that a simple first-order treatment of hyperfine interactions was inadequate to explain the observations, and an analysis following the theory of McConnell et al. (9) was used to verify the radical assignment and hyperfine coupling constants at all crystal orientations in the magnetic field.

EXPERIMENTAL PROCEDURE

Preparation of Crystals

Orotic acid was obtained commercially and used without further purification. This compound is only sparingly soluble in water, and our attempts to grow good single crystals from dilute aqueous solutions were unsuccessful. After trying several solvents, we found that a mixture of ethanol and NH$_4$OH was suitable for crystal growth. A solution of 50% ethanol and 50% 0.8 M aqueous NH$_4$OH (by volume) was saturated with orotic acid at 60°C. Slow cooling over a period of several days yielded large crystals with well-developed faces. These crystals showed no decomposition in air after harvesting.

In order to ascertain that the crystals obtained by this procedure were orotic acid, a crystal was dissolved in water and analyzed by ultraviolet (uv) spectroscopy and thin-layer chromatography. The uv absorption spectra of the dissolved crystal and of a solution of the commercial preparation were identical, and the absorbancy ratios at 280/260 and 250/260 agreed with those reported in the literature for orotic acid (10). Ascending chromatography on Eastman thin-layer 6065 (cellulose with fluorescence indicator) was carried out, using four solvents. With isopropanol: 30% HCl: H$_2$O (65:10:25), the Rf values for the dissolved crystal and the commercial preparation were 0.68 and 0.66 respectively; with isopropanol: H$_2$O (50:50), the respective values were 0.18 and 0.18; with methanol: 30% HCl: H$_2$O (70:20:10), the values were 0.52 and 0.50; and with n-butanol:methanol: H$_2$O: 0.8 M NH$_4$OH (59:20:20:1), the values were 0.22 and 0.25. All proportions given here are by volume.

To our knowledge, no report of the crystal structure of orotic acid appears in the literature. An orthogonal a'b'c' axis system, shown in relation to the crystal morphology in Fig. 1, was used to facilitate the ESR analysis.
These axes were found to be axes of magnetic symmetry. Specifically, in the a'b' plane, the spectrum at any angle $\theta$ relative to a' (or b') is identical to that at an angle $-\theta$ with that axis. There are two magnetically distinct molecules in the a'b' plane. In the b'c' and c'a' planes, all molecules are magnetically equivalent. The orientation of the molecules relative to the a'b'c' axis system will be inferred later from the ESR analysis.

**Irradiation**

The crystals were irradiated at room temperature with 7-MeV electrons from a linear accelerator. A total exposure of 10 megagerontgens was delivered at a rate of approximately 1 MR per minute. The radicals produced were quite stable, showing no appreciable decay or conversion at room temperature for several weeks.

**ESR Spectroscopy**

It was necessary to make ESR measurements at two microwave frequencies in order to analyze completely the hyperfine interactions observed. Data were taken at 9.4 GHz, using a Varian 4502 spectrometer, and at 16.2 GHz, using a microwave bridge constructed in our laboratory. In both cases, a cylindrical cavity was used to allow rotation of the magnet about the crystal. Hyperfine couplings were measured by comparison with a standard marker of Mn$^{++}$ in MgO. DPPH was used as a standard for determining g-values.

All calculations were made using a CDC 6600 computer.

**IDENTIFICATION OF THE RADICAL**

The spectra observed for many crystal orientations consisted of a hyperfine doublet with each component further split into an equal-intensity triplet. Figure 2 shows this basic pattern at four orientations in the b'c' plane. It is apparent that the unpaired electron interacts with one proton ($I = 1/2$) and one $^{14}$N nucleus ($I = 1$) to give this hyperfine structure. Both the nitrogen and hydrogen hyperfine couplings showed considerable anisotropy. The nitrogen coupling had its maximum value with the magnetic field along the c' axis and was too small to be resolved everywhere in the a'b' plane. This suggests that the unpaired spin density producing the nitrogen coupling is in a nitrogen $2p_{\pi}$ orbital whose direction is along c'. The c' axis was found to be the direction of minimum g-value, further substantiating this assignment. The hydrogen coupling was typical of that expected for coupling of unpaired spin density in a carbon $2p_{\pi}$ orbital to an $\alpha$ proton. Furthermore, the intermediate principal value for the hydro-
Table I. Principal values of the spectroscopic splitting tensor \( g(x) \), the nitrogen hyperfine coupling tensor \( A^{(i)} \), and the direction cosines relating the corresponding principal axes to the \( a'b'c' \) coordinate system. (Plus and minus signs for the direction cosines are for the two magnetically distinct molecules in the unit cell.)

<table>
<thead>
<tr>
<th>Principal value</th>
<th>Direction cosines</th>
</tr>
</thead>
<tbody>
<tr>
<td>( g_u )</td>
<td>2.0050 1 0 0</td>
</tr>
<tr>
<td>( g_v )</td>
<td>2.0052 0 1 0</td>
</tr>
<tr>
<td>( g_w )</td>
<td>2.0023 0 0 1</td>
</tr>
<tr>
<td>( A_{N}^{(a)} )</td>
<td>15.0 0 0 1</td>
</tr>
<tr>
<td>( A_{N}^{(b)} )</td>
<td>0 1 to (0, 0, 1)</td>
</tr>
<tr>
<td>( A_{N}^{(c)} )</td>
<td>7.3 ±0.94 0.34 0</td>
</tr>
<tr>
<td>( A_{N}^{(d)} )</td>
<td>±0.34 0.94 0</td>
</tr>
<tr>
<td>( A_{N}^{(e)} )</td>
<td>15.1 0 0 ±1</td>
</tr>
</tbody>
</table>

Nitrogen Hyperfine Interaction

Within experimental error, the nitrogen hyperfine tensor is axially symmetric, with the two degenerate principal values being zero. The general expression for the hyperfine coupling at an arbitrary angle in the principal axis system is

\[
A = (A_{x}^{2} \cos^{2} \theta_{x} + A_{y}^{2} \cos^{2} \theta_{y} + A_{z}^{2} \cos^{2} \theta_{z})^{1/2},
\]

where \( A_{x}, A_{y}, A_{z} \) are the principal values and \( \theta_{x}, \theta_{y}, \theta_{z} \) the corresponding direction cosines relating the direction of observation to the principal axes. Here, this expression reduces to

\[
A = (A_{N}^{2} \cos^{2} \theta)^{1/2},
\]

with \( \theta \) being the angle between the field direction and the symmetry axis of the nitrogen hyperfine tensor. In Fig. 4, the observed values for the nitrogen couplings are compared with the calculated values, based on parameters given in Table I.

Hydrogen Hyperfine Interaction

Ordinarily, the interaction of a single proton with the unpaired electron in a free radical splits the original absorption line(s) into a simple doublet. The two lines of the hyperfine doublet correspond to the two possible spin states \( M_{I} = ±1/2 \) for the proton, the selection rule being that \( M_{I} \) does not change its value simultaneously with the change in the electron spin state. This situation prevails if the
Fig. 3. Plot of the g-value in the three orthogonal planes of the a'b'c' axis system. Solid lines were calculated from tensor parameters in Table I; circles are experimental values. In the a'b' plane, solid and open circles are for the two magnetically distinct molecules in the unit cell.

Fig. 4. Plot of the nitrogen hyperfine coupling, as described in Fig. 3. No plot is shown for the a'b' plane, since the coupling was zero at all angles.

**FREE RADICALS IN PYRIMIDINES**

external magnetic field of the spectrometer is either much greater than or much less than the magnetic field produced by the electron at the site of the proton. In the former case, the proton is aligned either parallel or antiparallel to the external magnetic field, and its alignment is not expected to change during the electronic transition. In the latter case, the proton is aligned either parallel or antiparallel to the electronic magnetic field, and the electronic transition merely reverses the direction of this field. The proton spin state again is not expected to change, as it will still be quantized in the field.

A more complicated situation results when the external field is comparable in magnitude to the electronic field at the proton. In this case, the proton is oriented in the resultant of
the external and electronic magnetic fields. When the electronic transition occurs, the field at the proton neither remains fixed nor reverses, but instead takes on some intermediate direction relative to the initial resultant field. The selection rule restricting a change in the proton spin state no longer applies, since the proton must now become oriented either parallel or antiparallel to the new resultant field. Thus, four transitions become allowed rather than two. These four transitions comprise two pairs, referred to as the inner and outer doublets, each symmetrically disposed about the center of the original absorption line(s). The relative intensity of the two doublets depends on the angle between the resultant fields at the proton for the two electron spin states.

There are in general three unique orientations of a radical in the external field for which the proton hyperfine pattern is only a doublet even though the external field and the electronic field are comparable in size. These are called the canonical orientations, the directions in the radical being the principal hyperfine axes. For each of these orientations, the electronic field at the proton is parallel (or antiparallel) to the external field, so that the direction of the resultant field either remains the same or reverses during the electronic transition. Thus, the proton remains quantized, and the selection rule restricting a change in its spin state applies.

According to the theory of McConnell et al. (9), the resultant magnetic field at the proton, due to both the external and electronic fields, is in the direction of a unit vector \( \mathbf{u} \) given by an equation of the form

\[
Au = \frac{g_e \beta}{g_B} H_H - M_S \left( \mathbf{A}_x \sin \theta \cos \phi + \mathbf{A}_y \sin \theta \sin \phi + \mathbf{A}_z \cos \theta \right). \]

In this equation, \( g \) and \( g_e \) are the electronic and nuclear \( g \)-factors respectively, \( \beta \) is the Bohr magneton, and \( \beta_n \) is the nuclear magneton; \( u_H \) is a unit vector in the direction of the external magnetic field, and \( \mathbf{A}_x, \mathbf{A}_y, \mathbf{A}_z \) are unit vectors in the directions of the principal hyperfine axes. The corresponding principal hyperfine values being \( A'_x, A'_y, A'_z \) and \( A''_x, A''_y, A''_z \). \( \theta \) and \( \phi \) are the polar and azimuthal angles relating the external field direction to the principal axis system. Thus, this equation may be evaluated for the two electron spin states \( M_S = \pm 1/2 \) to give two values, \( u' \) and \( u'' \), for the direction of the resultant field at the proton. Similarly, two corresponding values of \( A \) are obtained, \( A' \) and \( A'' \). These parameters are then used to give both the splittings of the inner and outer doublets and their relative intensities. The inner doublet splitting equals \( A' - A'' \) and the outer doublet splitting equals \( A' + A'' \). The angle (\( \xi \)) between \( u' \) and \( u'' \), given by \( \cos \xi = u' \cdot u'' \), determines the relative intensities of the inner and outer doublets. For the inner doublet, the normalized relative intensity is \( \cos^2 (\xi/2) \), and for the outer doublet it is \( \sin^2 (\xi/2) \).

The theory discussed above was used to analyze the proton hyperfine coupling for the free radical in irradiated orotic acid. It can be seen that both the inner and outer doublet splittings, as well as their relative intensities, depend on the magnitude of the external field. In Fig. 5, this behavior is seen for a crystal orientation with \( H \) along the axis where all molecules in the unit cell are magnetically equivalent. At a microwave frequency of 9.4 GHz (\( H \approx 3,330 \) gauss), the inner and outer doublets are of comparable intensity, whereas at 16.2 GHz (\( H \approx 5,770 \) gauss), the inner doublet is much stronger than the outer doublet. At other crystal orientations, the outer doublet was more intense than the inner doublet. The spectra at both microwave frequencies...
are expected to be identical only when the crystal is in a canonical orientation, with \( H \) along a principal hyperfine axis.

In order to apply the theory of McConnell et al. (9) it is necessary to determine the principal hyperfine values \( A_x', A_y', A_z' \) and the directions of the principal axes relative to the crystal axis system. In this case, the intermediate hyperfine value can be measured directly as the splitting when \( H \) is along \( c' \), since this direction is parallel to the \( p \)-orbital (see earlier discussion). The other two principal values are then found as the maximum and minimum splittings in the \( a'b' \) plane. Unfortunately, there are two magnetically distinct molecules in this plane, and a canonical orientation for one is not a canonical orientation for the other. Nevertheless, it was possible to obtain the tensor parameters for the hydrogen hyperfine interaction, and these are given in Table I. The principal hyperfine axes for the two molecules are approximately 40 deg apart.

With these parameters, it should be possible to explain both qualitatively and quantitatively the spectra observed at all crystal orientations. This was found to be the case, and is considered as justification for the radical assignment and the tensor parameters in Table I. The spectra in Fig. 5 agree well with the theoretical lines at both microwave frequencies. Figure 6 shows spectra at another orientation where there are two magnetically distinct molecules. Again, good agreement is seen between the calculated and observed spectra. Quantitatively, the splittings of the inner and outer doublets were predicted well by the theory. A comparison between the observed and calculated splittings for the two doublets, with the magnetic field in the \( a'b' \) plane, is shown in Figs. 7 and 8 for the two microwave frequencies. In this plane, the nitrogen hyperfine coupling was zero, so the proton coupling could be analyzed more carefully than in the other two planes. The theoretical lines are shown as dashed lines in regions where the normalized relative intensity for a particular doublet falls below 0.30.

**CALCULATION OF SPIN DENSITIES**

The radical in irradiated orotic acid is formed by the same mechanism as one of the...
radicals in irradiated cytosine (6), and the spin densities at \( N(1) \) and \( C(5) \) can be compared for these two cases. Following the analysis used by Cook et al. (6) for cytosine, the spin density \( \rho_N \) in the \( 2p\pi \) orbital on \( N(1) \) may be calculated from the expression

\[
A_d = B_0 (3 \cos^2 \theta - 1) \rho_N
\]

where \( A_d \) is the dipolar component of the nitrogen coupling and \( B_0 \) is a constant equal to 17.1 gauss. The maximum value of \( A_d \) occurs when \( H \) is along the nitrogen \( 2p\pi \) orbital, so that \( \theta = 0 \) and \( A_d = 2B_0 \rho_N^0 \). In the present case, the nitrogen coupling is composed of both isotropic and anisotropic components, with the isotropic component of \( 1/3 (A_x + A_y + A_z) \) being equal to \( 1/3 A_{||} \), or 5 gauss. Thus, with \( \theta = 0 \), \( A_d = 10 \) gauss and \( \rho_N = 0.29 \). This value is exactly the same as that reported for the spin density on \( N(1) \) in cytosine (6).

The spin density on \( C(5) \) \( (\rho_C) \) may be determined from the hydrogen hyperfine interaction. Here, the isotropic component of the coupling \( (A_f) \) is related to \( \rho_C \) by the formula of McConnell and Chestnut (11),

\[
A_f = Q \rho_C
\]

where \( Q \) is an empirical constant. With the value of \( Q \) used for cytosine, and with the principal values in Table I, \( \rho_C \) is found to be 0.64. In cytosine, the spin density on \( C(5) \) was found to be 0.72.

In comparing the radicals in these two compounds, it should be noted that, in cytosine, an
additional spin density of 0.15 resides on N(3)' due to the resonance structure.

This is not possible in orotic acid if this compound is in the diketo form, as is usually the case with uracil derivatives. It seems somewhat surprising that the spin density on C(5) is larger in cytosine than in orotic acid, since cytosine has the additional site for spin density on N(3)'. It may be that the difference is due to the different groups on C(4) in the two compounds. In orotic acid, some additional spin density may reside on O(4)' due to the resonance structure and this would not be possible in cytosine.

There is, of course, no evidence for spin density on the oxygen atom, since the 16O nucleus has no magnetic moment.

MOLECULAR ORIENTATION IN THE CRYSTAL

Since the crystal structure of orotic acid is apparently not known, it is of value to predict the orientation of the molecules in the crystal from the ESR analysis. This can be done on the assumption that the radicals in the crystal do not reorient following hydrogen abstraction from N(1). First, from the direction of the minimum g-value and the maximum nitrogen hyperfine coupling, it can be concluded that the pyrimidine ring lies very nearly, and probably exactly, in the a'b' plane. This is the case for all molecules in the unit cell. In the a'b' plane, some additional information regarding the molecular orientation can be obtained from the directions of the hydrogen principal hyperfine values. Theoretically (12), the minimum principal value for an α proton should occur with the magnetic field along the C-H bond, and the maximum value in a direction perpendicular to both the C-H bond and the carbon p-orbital. Thus, these molecular directions can be related to the a' and b' axes. Due to the asymmetry of the orotic acid molecule, there are two possible orientations which will satisfy the conditions, these corresponding to a rotation of 180 deg about the C-H bond. Figures 9(a) and 9(b) show the two possible orientation schemes. In each case, two molecules are shown, corresponding to the two magnetically distinct species observed in this plane. It seems likely that there are four molecules in the unit cell of orotic acid, the two additional ones being related to the other two by centers of symmetry. These would not appear as distinct species in ESR data, however, since two molecules related by a center of symmetry are magnetically equivalent. It should be noted that the ESR parameters only indicate the probable orientation of the molecules within the crystal, and give no information on the spatial arrangement of the molecules.
ACKNOWLEDGMENTS

This research was supported by AEC Contract W-7405-eng-48, NASA's Bioscience Program, and AEC Contract AT(30-1)-3799.

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Paul Karl Horan is a National Institute of Health predoctoral trainee from Pennsylvania State University.
Irradiation of Dihydrothymine in Frozen Sulfuric Acid Solutions

Thormod Henricksen and Wallace C. Snipes

SUMMARY

Frozen solutions of dihydrothymine in H₂SO₄, irradiated at 77°K, contain large amounts of thymine after melting. Concentration studies show that the thymine must be produced mainly by indirect mechanisms. Low-temperature uv absorption measurements reveal that thymine is not present after irradiation at 77°K, but is formed upon warming of the samples to about 160° to 200°K.

ESR experiments suggest that the 5-thymyl radical is an intermediate in the production of thymine. At 77°K the irradiated samples contain only H atoms and some other unidentified H₂SO₄ radicals. The 5-thymyl radicals are formed upon warming to temperatures above 105°K, and they disappear in the temperature range at which thymine is formed.

It is well known that most radiation-induced reactions are slowed down or stopped entirely in frozen solutions at low temperatures. The freezing technique has even been used to distinguish between the direct and indirect actions of radiation, since in the frozen state the diffusion of water radicals seems to be very limited. The reduced ability of the water radicals to react with solute molecules in frozen solutions was demonstrated in some early electron spin resonance (ESR) experiments on sulfur compounds (1). Thus, it was shown that the OH radicals trapped during irradiation at low temperatures disappeared, upon warming, mainly in combination reactions. However, in more recent experiments on frozen acid and alkaline solutions, evidence has been presented for a much larger effect of the solvent radicals (2). It has been reported that both H atoms (3) and electrons (4) react with the solvent molecules.

In this paper experiments are presented which show an extremely large indirect radiation effect in frozen sulfuric acid solutions of dihydrothymine, the end product observed being thymine. An attempt has been made, by a combination of ESR technique and low-temperature ultraviolet (uv) absorption measurements, to follow the sequence of reactions giving rise to the large indirect effect. In particular, the experiments were designed to establish whether the indirect effect occurs at the irradiation temperature (77°K) or during the subsequent warming and melting of the solutions.

EXPERIMENTAL PROCEDURE

Sample Preparation and Irradiation

Sulfuric acid solutions ranging from 0.2 to 9.0 mole/liter were used. The dihydrothymine concentration varied from 0.025 to 4.0 mg/ml (i.e., 1.95 × 10⁻⁴ to 3.12 × 10⁻² mole/liter).
Most experiments were carried out with a dihydrothymine concentration of 1.0 mg/ml ($7.8 \times 10^{-3}$ mole/liter).

Samples of approximately 3 ml were rapidly frozen to $77^\circ$K in glass test tubes. In the concentration range from approximately 4 to 8 M H$_2$SO$_4$, rapid freezing usually results in glassy samples. The glassy samples turn polycrystalline if kept for some time at a higher temperature (see below).

The samples were irradiated at $77^\circ$K with 6.5-MeV electrons from a linear accelerator. Doses ranging from 0.05 to 40 MR were delivered at a rate of approximately 1 MR/min.

After irradiation, the samples were melted and the uv absorption was measured with a Cary 14 recording spectrophotometer. The intensity of the absorption peak at 265 nm was used for a quantitative determination of the amount of thymine produced. Corrections were made for the small absorption of dihydrothymine at this wavelength.

**Low-Temperature uv Absorption Measurements**

Whereas the quantitative determination of the thymine formation was carried out on melted samples, efforts were made to observe directly the uv absorption of frozen samples at different temperatures. For this purpose a Cary Low-Temperature Dewar, model 1440650, was used. Solutions of 5 M H$_2$SO$_4$, with or without dihydrothymine, were carefully frozen in quartz tubes to give clear, glassy samples. The solutions containing dihydrothymine showed less tendency to crack than the pure solvent. Only those samples with a minimum of cracks were used, since any imperfection or crystallinity interferes with the uv absorption measurements.

The samples were irradiated through a quartz window in the Dewar, oriented at 90 deg from the light beam of the spectrophotometer. This arrangement prevented radiation-induced absorption in the surfaces of the sample holder through which the light beam passed.

A copper-constantan thermocouple placed on the outside of the sample holder gave an indication of the approximate sample temperature. The irradiation temperature was about 88$^\circ$K, and the subsequent absorption measurements started at that temperature. By blowing nitrogen gas through the Dewar the sample temperature could be gradually increased. In most experiments the temperature increased by approximately 2.5$^\circ$/min, and absorption spectra were taken continually while the sample was warming. At a temperature of about 185 to 190$^\circ$K the glassy samples started to turn polycrystalline, thus preventing further absorption measurements.

**ESR Measurements**

ESR samples were made from 5 M H$_2$SO$_4$ solutions. Small drops were rapidly frozen at $77^\circ$K to give glassy samples. These were evacuated and sealed in quartz tubes. After irradiation, ESR spectra were recorded at $77^\circ$K with a Varian 4502 X-band spectrometer. The samples were heat-treated in a Varian Variable Temperature Accessory outside the cavity, then returned to $77^\circ$K for further measurements. The relative quantitative data are taken from the amplitudes of certain peaks in the first- or second-derivative spectra. No attempts were made to obtain absolute yield data.

**RESULTS AND DISCUSSION**

**Results on Melted Samples**

It has been reported earlier that irradiation of dihydrothymine both in aqueous solutions and in the solid state yields thymine (5, 6). In the experiments described here, the uv-absorbing
IRRADIATION OF DIHYDROTHYMINE IN SULFURIC ACID

Fig. 1. Dose-response curve for the production of thymine in frozen solutions of dihydrothymine (1 mg/ml) in H$_2$SO$_4$ (3 M). The samples were irradiated at 77°K, then melted for assay of thymine. The thymine concentration was determined by the absorbance at 265 nm. The results for the low-dose region are shown by the inset.

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substance produced upon irradiation in the frozen solutions had an absorption spectrum identical to that of thymine, and in all subsequent calculations we assume that the product is thymine.

Figure 1 shows the dose-effect curve for the formation of thymine in a 3 M H$_2$SO$_4$ solution containing 1 mg/ml dihydrothymine. The solutions were irradiated at 77°K and the measurements carried out after melting. The thymine formation appears surprisingly large for irradiation in the frozen state. Thus, from the initial portion of the dose-effect curve (see inset of Fig. 1) it is seen that 0.07 mg/ml thymine is formed per MR. If it were assumed that only the energy absorbed by the dihydrothymine could lead to the production of thymine, this corresponds to a g-value (molecules thymine per 100 eV absorbed in dihydrothymine) of 535. This large value indicates some indirect effect, and the corresponding g-value based on the amount of energy absorbed in the entire solution is 0.47.

Similar results for some other H$_2$SO$_4$ concentrations are presented in Fig. 2. All dose-effect curves were found to be linear in the low-dose region. The g-values calculated on the basis of direct action alone are 33, 125, and 960 for 0.5, 1.0, and 6.0 M H$_2$SO$_4$ solutions respectively. Consequently, the conclusion is inescapable that in the frozen solutions a large indirect effect operates whereby radiation energy absorbed in the solvent can cause the production of thymine from dihydrothymine. Thus, subsequent g-value calculations will be based on the amount of energy absorbed by the entire solution.

Figure 3 shows the variation of g-value with H$_2$SO$_4$ concentration in the range 0.02 to 9.0 M.
The $H_2SO_4$ concentration is plotted logarithmically, giving a linear pH dependence for this plot. In experiments with dihydrothymine in pure aqueous solution, irradiation at 77°K with doses up to 25 MR gave no detectable amount of thymine.

Further evidence for the indirect action of radiation in the frozen solutions was obtained by investigating the dependence of the thymine formation on dihydrothymine concentration. The shape of the curve shown in Fig. 4 clearly demonstrates the indirect effect. Each point in this figure is based on a total dose-effect curve. In the sections that follow, experiments are described which give some information concerning the mechanism for the production of thymine by the indirect action of radiation.

**uv Absorption Experiments at Low Temperatures**

The above results show that large amounts of thymine are formed by indirect action when $H_2SO_4$ solutions of dihydrothymine are irradiated at 77°K. However, experiments in which the assay is carried out after melting cannot yield information as to whether thymine is formed during the exposure at 77°K or during the subsequent warming and melting of the samples. In order to determine which is the case, low-temperature uv absorption measurements were carried out on glassy 5 $M H_2SO_4$ solutions.

Figure 5 shows the uv absorption spectra of irradiated frozen 5 $M H_2SO_4$ solutions with and without dihydrothymine present. The spectrum of the unirradiated frozen solution has been subtracted from all the spectra in Fig. 5 to correct for the absorbance of the sample cell and the dihydrothymine at short wavelengths. During irradiation, the solutions turn yellow and exhibit a rather uniform absorption throughout the uv region. Both the color and uv absorption start to disappear at about 160°K and have vanished completely at about 185°K (Fig. 5b). The spectra of the dihydrothymine solutions show...
clearly that no thymine is formed during the exposure at approximately 88° K. Furthermore, the spectra in Fig. 5(a) demonstrate that thymine is indeed formed in the frozen solutions upon warming.

It is possible to obtain from the UV absorption spectra an estimate of the thymine formation at the different temperatures. This may be expressed as percent of the final amount of thymine present after melting, according to the formula

\[
\frac{A_{\text{sol}(T)} - A_{\text{sol}(295\degree K)}}{A_{\text{sol}(295\degree K)}} \times 100,
\]

where \(A_{\text{sol}(T)}\) and \(A_{\text{sol}(295\degree K)}\) are the 265-nm absorptions of the dihydrothymine solution and the pure solvent, respectively, at temperature \(T\), and \(A_{\text{sol}(295\degree K)}\) is the absorption of the dihydrothymine solution after melting. The results for three different experiments using 5 M \(\text{H}_2\text{SO}_4\) and 1 mg/ml dihydrothymine are presented in the right-hand portion of Fig. 6. The three curves do not coincide exactly, probably due to small variations in the warming rate and errors in temperature measurement (the thermocouple was on the outside of the sample tube). However, the curves show that in these solutions thymine is formed in a rather narrow temperature range beginning at about 160 to 170° K and extending up to about 200° K. It should be emphasized that the samples turned polycrystalline at about 190° K in these experiments and no absorption measurements could be made between this temperature and the temperature at which the samples melted.

**ESR Measurements**

All the experiments presented above have concentrated on the formation of thymine as measured by its absorption at 265 nm. It is
Fig. 6. The appearance and disappearance of some of the species induced in 5 M H$_2$SO$_4$ solutions of dihydrothymine versus the sample temperature. For the free-radical measurements (ordinate to the left), the samples were irradiated at 77°K and then stepwise heat-treated for 4 min at the temperatures indicated. All observations were made at 77°C. For the uv-absorption measurements of thymine (ordinate to the right) the samples were irradiated at 88°C and subsequently warmed at a rate of approximately 2.5°C/min. The 265-nm absorption was measured at the temperatures indicated. All quantities in this figure are relative and given as % of the maximum value obtained for the particular species (i.e., the absolute values of H atoms and 5-thymyl radicals are different).

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evident that a large indirect effect operates and that the secondary reactions take place between 77 and 200°C. In the radiation chemistry of pyrimidines it appears that the indirect action is mediated through free-radical reactions at the 5, 6 double bond (for a review see Ref. 5), but the intermediates are too transient to be observed by ESR without some trapping technique. However, in these experiments on frozen solutions free radicals are trapped and may therefore be studied by ESR.

Figure 7 shows the ESR spectra for a glassy 5 M H$_2$SO$_4$ solution containing 3 mg/ml dihydrothymine. Following irradiation at 77°C, the

characteristic 506-G hyperfine doublet of hydrogen atoms is seen. The central part of the resonance, which here is entirely due to radicals induced in the solvent, has tentatively been ascribed to SO$_4^-$ and SO$_3^-$ radicals (7, 8), but so far no ESR evidence has been presented for this interpretation. Upon heat treatment the hydrogen lines disappear and additional absorption lines are seen on either side of the
central resonance. These lines were found to be due to the 5-thymyl radical, which has a characteristic eight-line hyperfine pattern spreading over approximately 140 G when randomly oriented. The outer components of this pattern are sufficiently separated from the central H$_2$SO$_4$ resonance that the formation of the 5-thymyl radical at different temperatures can be studied. In Fig. 7 (lower part) are presented spectra taken after heat treatments for 4 min at increasing temperatures. The sweep rate and the sensitivity are different from those for the top spectrum. In the first place, it can be concluded that very few if any 5-thymyl radicals are formed at 77°K. Secondly, the 5-thymyl radicals, which appear after heat treatment, are stable up to about 160°K, at which the remaining H$_2$SO$_4$ radicals have disappeared. Upon further heat treatment at 175°K, more than 90% of the 5-thymyl radicals have disappeared.

Some quantitative data for the appearance and disappearance of the 5-thymyl radicals are given in Figs. 6 and 8. For comparison, the disappearance of the hydrogen atoms is also given. It seems that the 5-thymyl radical disappears in the same temperature region where thymine is formed, suggesting that the radical is an intermediate in the sequence of events whereby dihydrothymine is converted into thymine in the irradiated frozen H$_2$SO$_4$ solutions. The sequence of events may therefore be

Here R is a radical from the solvent. It is not clear whether some additional species are involved in the second reaction or whether it occurs spontaneously. If hydrogen atoms are released from the 5-thymyl radical when thymine is formed these could undergo combination reactions to produce H$_2$.

The data in Figs. 6 and 8 also suggest that the radical R may in part consist of a hydrogen atom. Thus, a certain fraction of the 5-thymyl radicals (approximately 50% according to Fig. 8) are formed concomitantly with the disappearance of the hydrogen atoms. This indicates that hydrogen atoms may react with dihydrothymine when they become released from their trapping sites. This mechanism is supported by the results on neutral solutions. Thus, no hydrogen atoms and few if any 5-thymyl radicals were trapped when pure aqueous solutions were irradiated at 77°K and heat treated. Likewise, no thymine was produced even after 25-MR irradiation. The hydrogen atoms are in turn
formed by reactions of H⁺ ions with electrons when they become thermalized as proposed previously (7, 9). It should, however, be pointed out that the H atoms could not be the only precursor radicals for the 5-thymyl radical, since a large fraction of the latter is formed subsequent to the disappearance of the hydrogen atom doublet.

ACKNOWLEDGMENT

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REFERENCES AND NOTE


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Subfractionation of the Low Density Sf 0-20 and High Density Lipoproteins

Frank T. Lindgren, Lin C. Jensen, Robert D. Wills and Gary S. Stevens

SUMMARY
Subfractionation of the low-density Sf 0–20 and high-density plasma lipoproteins has been accomplished by using a cumulative flotation rate procedure. Fractionation employs nonlinear salt gradients and high-performance swinging-bucket rotors. Physical and chemical data on LDL subfractions are in general agreement with earlier data. Lower molecular weight data are obtained for HDL subfractions than reported earlier; however, this may be the result of the different fractionation procedures used.

Plasma low density lipoproteins (LDL) of the Sf 0-20 class previously have been subfractionated on density gradients (1-3). These studies have shown that with increasing Sf rate, the molecular weight increases and hydrated density decreases. The change in hydrated density appears to be primarily the result of changes in protein content. Similarly, fractionation of the high density lipoprotein (HDL) spectra on the basis of density (4) has usually given two fractions, HDL2 (1.063 < σ2 < 1.125) and HDL3 (1.125 < σ3 < 1.20). Again, the content of protein appears to be significantly greater (5, 6) in the more dense lower-molecular-weight (7, 8) HDL3 fraction.

Previous methodology usually has involved either equilibrium density gradient procedures or two or more preparative stages, each requiring density manipulation. The present method is an extension of a cumulative flotation rate procedure described earlier (9, 10) for the Sf > chylomicrons and the Sf 20–400 very-low-density lipoproteins (VLDL). The availability of high-performance long-radial-path swinging bucket rotors allows extension of this method to the much lower-molecular-weight LDL and HDL spectra.

METHODS
Before the LDL subfractionation, Sf > 20 lipoproteins must be first removed from plasma. Two-ml aliquots of plasma are each mixed with 4 ml of 0.195 M NaCl (containing 10 mg/100 ml EDTA). Six of these 6-ml mixtures are centrifuged at 40,000 rpm at 18°C for 18 hr in a 40.3 Spinco rotor. The Sf > 20 lipoproteins are removed with a capillary pipette (i. d. = 0.5 mm) and the bottom 2 ml of each preparative tube pooled. The small-molecule background density of this Sf < 20 plasma is raised to a density 1.118 g/ml by adding 2080 mg NaCl to 12 ml of this bottom fraction. (Unless otherwise indicated densities are given at 20°C.) Two-ml aliquots of this Sf < 20 plasma are overlayed with a sodium chloride gradient in six 9/16-in. o. d. X 3.5-in. preparative ultracentrifuge tubes. The solutions (from high-
For subfractionation of the HDL spectra the total LDL, $S_f < 1.065$ g/ml must be removed first. For this purpose and to achieve concentration of the HDL, three 4-ml aliquots of serum are mixed with 2 ml of a solution that is 0.199 M in NaCl and 2.43 M in NaBr (p = 1.1815 g/ml) which contains 10 mg/100 ml EDTA. After centrifugation for 24 hr at 18°C in a 40.3 rotor, the concentrated HDL are removed in the 3.5–5.5-ml layer. To 6 ml of this fraction 3022 mg of solid NaBr is added, bringing the density to approximately 1.395 g/ml. One-ml aliquots of this HDL-containing fraction are placed in 0.5×2.5-in. tubes and overlaid with an approximate equilibrium NaBr density gradient. Two 0.5-ml solutions of 1.3622 and 1.3424 g/ml are first added. Then two 1-ml solutions of 1.3161 and 1.2856 g/ml are added, and finally, two 1.5-ml solutions of 1.2521 and 1.1973 g/ml
SERUM HDL SUBFRACTIONATION, F_{1.20} 0-9

7 ml NaBr gradient, SW 45 rotor, 23°C

- RUN I, $236.6 \times 10^6 \text{g} \times \text{min}$
  (20h-52.8m UTS, 42,000 RPM)
- RUN II, $287.6 \times 10^6 \text{g} \times \text{min}$
  (24h-13.1m UTS, 43,000 RPM)

Fig. 2. Sodium bromide density gradient used for HDL subfractionation. All NaBr solutions have a constant 0.195 M NaCl content.

ANTICIPATED RECOVERY
After lipoproteins < 1.063 g/ml are removed

Fig. 2 shows the corrected schlieren plots of each subfraction from each of the subjects. The observed recoveries agree with that expected from calculation. A summary of the physical and chemical data is shown in Table I. These data show that with increasing $S_f^0$ rate, molecular weight increases and both hydrated density and protein content complete the 7-mI density gradient. The details of this gradient and expected recovery are shown in Fig. 2.

$S_f^0$ (and $F_{1.20}^0$) rates, molecular weights, and hydrated densities are calculated by an $\eta F^0$ versus $\rho$ plot, as described in detail elsewhere (3). Flotation rates for the low density fractions were measured at approximately 1.061 and 1.098 g/ml. Flotation rates for the HDL fractions were measured at approximately 1.200 and 1.286 g/ml. Solid NaCl or NaBr was added to appropriately raise the density of the low- and high-density fractions, respectively. In all cases, densities and density manipulation were monitored by precision refractometry.

Total lipoprotein mass was determined by NCH elemental analysis (11) and the known elemental composition of the lipoprotein. From a total lipoprotein elemental phosphorus determination (12) and the N/NCH elemental ratios, protein content of each lipoprotein fraction was determined (40). All elemental measurements were duplicate analyses using a modified Model 185 CHN analyzer (Hewlett-Packard, Palo Alto, California).

RESULTS AND DISCUSSION

LDL subfractionation was performed on plasma from two fasting patients, clinically characterized as type IV hyper-pre-β-lipoproteinemia (13). Figure 3 shows the corrected schlieren plots of each subfraction from each of the subjects. The observed recoveries agree with that expected from calculation. A summary of the physical and chemical data is shown in Table I. These data show that with increasing $S_f^0$ rate, molecular weight increases and both hydrated density and protein content
Table I. LDL subfractionation, type IV subjects.

<table>
<thead>
<tr>
<th>Case</th>
<th>S&lt;sub&gt;f&lt;/sub&gt; Range</th>
<th>ρ Inter (g/ml)</th>
<th>Peak S&lt;sub&gt;f&lt;/sub&gt; Rate (svedbergs)</th>
<th>Mol. Wt. (millions)</th>
<th>Protein (wt %)</th>
<th>PL (wt %)</th>
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<td>(I)</td>
<td>876 10.4-20.0</td>
<td>1.0079</td>
<td>13.5</td>
<td>3.04</td>
<td>16.9</td>
<td>24.8</td>
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<tr>
<td></td>
<td>877</td>
<td>1.0110</td>
<td>13.5</td>
<td>3.34</td>
<td>17.9</td>
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<tr>
<td>(II)</td>
<td>876 5.7-12.0</td>
<td>1.0272</td>
<td>6.89</td>
<td>2.17</td>
<td>22.6</td>
<td>23.3</td>
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<tr>
<td></td>
<td>877</td>
<td>1.0281</td>
<td>6.87</td>
<td>2.25</td>
<td>23.8</td>
<td>22.6</td>
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<tr>
<td>(III)</td>
<td>876 3.5 - 6.5</td>
<td>1.0348</td>
<td>4.96</td>
<td>1.91</td>
<td>25.5</td>
<td>22.3</td>
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<tr>
<td></td>
<td>877</td>
<td>1.0393</td>
<td>4.13</td>
<td>1.88</td>
<td>26.6</td>
<td>21.2</td>
</tr>
</tbody>
</table>

LDL SUBFRACTIONATION, Type IV subjects

Fig. 3. Corrected schlieren plots of LDL subfractions for subjects 876 and 877. Expected recovery is shown above.

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decrease, the latter confirming earlier data (1). Further, the range of molecular weight from 1.9-3.3 millions for LDL fractions whose major component varied from S<sub>f</sub> 4-13 agree generally with recent physical data on these lipoproteins (3, 14, 15).

HDL subfractionation was performed on plasma from two normal nonfasting adults. The corrected schlieren plots for the two subfractions from each subject are shown in Fig. 4. Again, observed recoveries agree approximately with calculated recoveries. The decision to achieve 100% recovery of F<sub>1.20</sub> 3.0 molecules was arbitrary. Actually, fractionation at F<sub>1.20</sub> 3.5 or 4.0 would probably yield subfractions somewhat closer in identity to the earlier HDL<sub>2</sub> and HDL<sub>3</sub> components. It should be observed that the earlier techniques fractionated primarily on the basis of density. In contrast, our procedure fractionates essentially on the basis of particle size, and therefore the two procedures may be achieving a different type of fractionation within the HDL spectra.

The physical and chemical data are summarized in Table II. These preliminary data show similar hydrated densities but lower molecular weights than earlier data, particularly for the less dense F<sub>1.20</sub> 3.0-9.0 components (6-8). These discrepancies may be, in part, the result of the different fractionation procedures used. However, chemical composition, and in particular protein content (44 to 55%), are in general agreement with the data based on the older HDL<sub>2</sub>-HDL<sub>3</sub> subfractionation. In order to compare procedures, it will be necessary in the future to determine the density heterogeneity of each subfraction acquired by "cumulative flotation rate" separations.
SUBFRACTIONATION OF $S_{0-20}$ AND LIPOPROTEINS

HDL SUBFRACTIONATION, Normal subjects

<table>
<thead>
<tr>
<th>Case</th>
<th>$F_{1.20}$ Range</th>
<th>$\sigma (\rho \text{Inter})$</th>
<th>Peak $F^*$ rate</th>
<th>Mol wt (thousands)</th>
<th>Protein (wt %)</th>
<th>PL (wt %)</th>
</tr>
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<tbody>
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<td>879</td>
<td>(2.7–9.0)</td>
<td>1.0988</td>
<td>4.30</td>
<td>265</td>
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<td>1.84</td>
<td>148</td>
<td>55.0</td>
<td>25.7</td>
</tr>
</tbody>
</table>

The earlier techniques have certain advantages in that the use of angle-head rotors simplifies methodology and at the same time permits fractionation on a larger scale. On the other hand, our procedure provides lipoprotein fractions in a media of defined small-molecule composition with minimal contamination from other plasma lipoproteins and proteins. There is also essentially no contamination from the plasma small-molecule components. Where contamination is important, as in immunological studies, such fractionation may help define more accurately the characteristics of the protein moiety within subfractions of all the plasma lipoproteins. In addition, the "cumulative flotation rate" procedure is flexible, allowing the collection of two or more subfractions of any desired flotation-rate interval within the important LDL and HDL spectra. Where very large fractions from a single plasma sample are needed, this general technique might be used in a zonal type rotor (16).
ACKNOWLEDGMENTS

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REFERENCES

The Glycoprotein Nature of the Smooth Membranes of Acid Secreting Cells

Gertrude M. Forte, Martin D. Pelavin and Robert M. Glaeser

SUMMARY

The apical plasma membrane, the internal smooth, tubular membrane, and the Golgi of acid-secreting cells were found to be highly reactive when stained with silver methenamine, which stains glycoproteins. The similarity in staining of the smooth internal membranes and apical surface membranes supports the hypothesis that the smooth membranes undergo transformations and become part of the surface membrane during HCl secretion. Studies on the presence of sialic acid in the membranes, to account for their stainability, showed there was no detectable sialoprotein in the smooth membrane cell fractions. Some sialic acid was present in the sedimenting fraction containing nuclei, cell envelopes, and mucus, but may represent contamination, since mucus is known to contain sialoprotein.

The oxyntic or acid-secreting cells of the frog stomach are characterized by abundant smooth-surfaced tubular structures within the apical cytoplasm. The membranous structures are, in fact, so numerous that they occupy at least 50% of the cell volume. These tubules have been shown (1, 2) to be involved in the process of HCl acid secretion. The nonsecreting oxyntic cells have relatively smooth lumenal surfaces. Actively secreting cells, on the other hand, have numerous long cytoplasmic extensions projecting from the lumenal surface, thus tremendously increasing the apical (or secretory) surface of the cell. This increase in area of the cell surface is believed to take place at the expense of the smooth tubular membrane system of the cytoplasm through membrane transformations, i.e., the internal smooth membranes move up to the apical surface and become confluent with it.

Although the fine structural changes strongly suggest intimate interrelations between the internal membrane system and the lumenal surface membrane, more precise chemical and cytochemical work is required to fully understand the nature of this relationship. In this study we have endeavored to histochemically identify glycoprotein substances in the oxyntic cells by the periodic acid-silver methenamine staining method (3), and relate this to the sialoprotein content of the smooth membranes. It has been suggested (3) that the silver methenamine staining of certain cytoplasmic membranes as well as the surface membranes may be attributable to sialic acid. It is known that isolated smooth membranes from guinea pig cerebral cortex (4), HeLa cells (5), and rat (6) and rabbit liver cells (7) contain sialic acid.
METHODS

Materials

Both leopard frogs, Rana pipiens, and bullfrogs, R. catesbeiana, were used in these studies. Cytochemical studies were carried out on R. pipiens. When cell fractionation studies were carried out, R. catesbeiana were used, since they yielded far more material.

Electron Microscopy

Tissue preparation: Gastric mucosal material was fixed for 90 min at room temperature in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The minced tissue was rinsed for 90 min in 0.1 M sodium cacodylate with three changes of buffer. Dehydration was carried out by rapidly carrying through graded alcohols (5-min periods were used). The tissue remained in propylene oxide–Epon for 3 to 4 hr and then was embedded in Epon and sectioned.

Section staining: The periodic acid–chromic acid–silver methenamine method for staining glycoproteins was employed as described in Ref. 3. Since we used Epon embedding material instead of Vestopal it was found necessary to stain the sections for 40 to 45 min in the silver methenamine solution.

Negative staining: Isolated smooth membranes (microsomes) were fixed several hours in the cold in 0.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. A small sample of fixed material was diluted with an equal amount of 2% sodium phosphate buffer, pH 7.5, and a droplet of this mixture was placed on a Formvar-carbon-coated grid. After 1 min excess fluid was removed with filter paper and the grid was immediately examined in the Hitachi HU 11 microscope.

Isolation of Subcellular Fractions

Excised stomachs of R. catesbeiana were used in all membrane-isolation studies. The opened stomachs were blotted several times, with mucosal surface down, on filter paper to remove adhering mucus and loose cells. The mucosal cells were then scraped from the underlying connective tissue layer with a stainless steel spatula. The scraped material from 4 to 6 stomachs was pooled and placed in a precooled Potter–Elvejhem-type homogenizer with a Teflon pestle. The first homogenization procedure used was that described in Ref. 8, utilizing a mannitol–sucrose–EDTA homogenization medium. It was found, however, that the sugars interfered with the colorimetric determination of sialic acid content.

In order to avoid introducing sugars into the homogenization medium, a solution consisting of 0.12 M KCl adjusted to pH 7.4 with 0.1 M Tris was used for the isolation procedure. This solution proved satisfactory for the isolation of the smooth, tubular membrane structures, since this fraction, when examined with the electron microscope, consisted of tubular structures very similar to those seen in the intact tissue.

Differential centrifugation steps for isolating various cell fractions are as outlined in the diagram.
Centrifugations were carried out in a Servall preparative centrifuge except for the isolation of Fraction III, which was accomplished with the Spinco Model L. All operations were carried out at 0 to 4°C.

The various fractions were identified as: Fraction I, containing nuclei, cell envelopes, and debris which appeared to be mucus; Fraction II, heavy microsomes; Fraction III, light microsomes.

Sialic acid determination

Warren's thiobarbituric acid analysis (9) for sialic acid was employed. Both H₂SO₄ and Clostridium perfringens neuraminidase (Worthington) were used to hydrolyze the smooth membrane fraction. The sodium m-periodate, cyclohexanone, and sodium arsenite were products of J. T. Baker Chemical; 2-thiobarbituric acid was obtained from Eastman Organic. N-acetylneuramic acid was used as a standard. The color produced in the assay was analyzed in the Cary-14 split-beam recording spectrophotometer.

RESULTS

Electron Microscopy

The morphology of oxyntic cells which have been fixed only in glutaraldehyde and embedded in Epon is seen in Fig. 1. The lumen (L) of the gastric gland is surrounded by portions of two cells. Many cytoplasmic extensions (MV) from the apical surface of the cell can be seen projecting into the lumen. Beneath the apical surface the greater part of the cell is packed with smooth membrane profiles of tubules, both in transverse and in longitudinal section. The packing formation is rather regular; one often sees groups of tubules arranged in a hexagonal array. The tubular diameter is fairly constant and is approximately 400 Å.

The mitochondria of the cell are mostly in the posterior region, and surround the nucleus. Unlike their structure seen after osmium fixation, the glutaraldehyde-fixed mitochondrial membranes appear in negative relief, since by this procedure the protein matrix of the mitochondria becomes more densely stained than do the cristae.

Periodic Acid-Chromic Acid-Silver Methenamine Staining

Sections which had been oxidized with peri-
periodic acid and chromic acid consistently showed the reduced silver pattern seen in Fig. 2. The surfaces of the oxytic cells are heavily stained and surface irregularities are clearly seen. The cytoplasm of the apical portion of the cell is also heavily stained; this is the region of the smooth internal-membrane tubules. On close examination of the micrograph one can see transverse profiles that are extremely electron-dense, and long strand-like dense structures, which represent the longitudinal aspects of the tubules. The Golgi apparatus, which is found basally and lateral to the nucleus, is also highly reactive. Mitochondria are almost electron transparent, and the nucleus, also, is almost devoid of stain. The lateral borders of the cell as well as the basal membrane and its infoldings are only slightly reactive. The tight junctions and desmosomes, however, do not appear to be stained. Indications are that this staining technique is very sensitive in localizing membrane glycoproteins.

Negative Staining

Microsomes that have been fixed and negatively stained are seen in Fig. 3. This frac-
Sialic Acid Determination

During the course of determining sialic acid content in the membrane fractions it was found that interfering chromophores appeared in the region between 530 and 550 mµ. The appearance of the chromophore made it extremely difficult to determine the absorbance of sialic acid, which absorbs at 549 mµ. The various reagents were tested for purity and it was found that cyclohexanone may be a source of impurities. Several sources of this reagent were tested; the J. T. Baker product produced the least masking effect. Further extraction with isobutanol did not improve the absorbance in the critical region.

Of the various membrane fractions tested (Fractions I, II, III) only Fraction I, which contained nuclei, cell membranes, and mucus debris, contained sialic acid. After hydrolysis with 0.1 M H2SO4, or after enzymatic treatment with neuraminidase, this fraction produced a large absorbance peak at 550 mµ. Fractions II and III (the microsomal fractions) had no detectable sialic acid when assayed under the same conditions as Fraction I.

DISCUSSION

The cytochemical silver staining reaction has been attributed to the reduction of silver methenamine by the aldehydic groups produced by the oxidation of 1,2-glycols and α amino alcohols by periodic and chromic acids (3). In an early work, Glegg, Clermont, and Leblond (10) have shown that the neutral sugars are readily stainable but the acid mucopolysaccharides such as heparin and chondroitin sulfate are unstainable. Application of the silver staining technique by Rambourg and Leblond (11) to numerous types of tissues revealed that the stain was most pronounced at the cell surface, on inner aspects of the Golgi, and in vesicular structures having their origin from the Golgi. From the results of in vitro studies on the stainability of various carbohydrates, these authors felt that glycoproteins are the most likely reactive groups in the stained cell structures. Furthermore, they have suggested that the glycoprotein may be sialoprotein. Certainly the appearance of sialic acid in surface membranes has been well documented (12), and it has been found in isolated smooth membranes from various tissues (5, 7, 13, 14).

Periodic acid–Schiff staining of acid-secreting cells from dog stomachs has been reported by Gerard et al. (15). Since there were no accompanying fine structural studies it was impossible to localize the site of the stain intracellularly. Recently, Sedar has shown (16) that a polysaccharide substance, probably a neutral carbohydrate, lines the smooth-surfaced membrane system and outer cell coat of parietal cells of the rat after phosphotungstic acid staining. The staining pattern which we have demonstrated in oxyntic cells of the frog is similar to that described by Sedar, since both the smooth cytoplasmic membranes and surface membranes are mutually reactive.

The fact that both the smooth cytoplasmic membranes and apical surface membranes have similar staining properties makes membrane transformations between these two components a more plausible hypothesis. The periodic acid–silver reactivity of the Golgi in oxyntic cells is of special interest. In a previous study on development of tadpole oxyntic cells we suggested that this organelle was the source of the smooth membranes found in mature oxyntic cells (17). Since both the Golgi and the smooth membranes are highly reactive, this interrelationship is far more tenable.
The function of the stainable layer on the membranes is as yet unknown. There are at least three possibilities, any or all of which may be involved: (a) the glycoprotein may act as a "recognition" factor at the fusion point to facilitate membrane transformations during active HCl secretion, (b) the carbohydrate may function as a protective barrier to prevent denaturation of membrane proteins during H⁺-ion production, (c) the carbohydrates may actually contribute directly to the transport and production of H⁺ ions.

Understanding the chemical nature of the silver-staining material found associated with the membranes is of extreme importance, since it would help us to understand the function of the glycoprotein layer. The lack of sialic acid in the smooth membrane fractions is highly interesting, since this glycoprotein has been shown to be a major component of microsomes from liver cells (7) and HeLa cells (5). In the study reported here sialic acid was found in Fraction I, containing nuclei, plasma membranes, and mucus. Since other investigators have shown that gastric mucus contains sialoprotein (15), it is likely that the sialic acid in Fraction I is due either partially or entirely to contaminating mucus. These negative results suggest that other sugars such as amino sugars may be the reactive groups, and work is presently underway to identify these substances.

REFERENCES
Information Content of Electron Microscope Images: 
Color and Depth Information as Vehicles for Subjective Information Transfer

James B. Pawley, Frank T. Upham, Alfred A. Windsor and Thomas L. Hayes

SUMMARY Subjective experiential modes of information transfer from scanning electron microscope image to observer can be added to the more traditional objective analytic modes. Color modulation and color-coding techniques offer one method for the study of subjective response in the observer.

It may be valuable to add subjective modes on information transfer to the usual objective or analytic modes. Subjective modes are not neat, and vary from person to person, but they can become increasingly important as the systems under study become more complex. By limiting ourselves to the digital information (words, numbers) that is the same for all people we would be neglecting a significant part of each individual. In order to utilize as much as possible of the entire person of the observer, we wish to build up an image that corresponds to experienced vision. Not all images contain the kinds of information necessary for this correspondence, and certainly not all information received via the eye can be compared to experienced vision. It is only when an image contains the parameters of experienced vision that the confidence associated with terms like "direct visualization" is justified. High resolution alone does not guarantee a useful image. We must also consider the kinds and levels of information transmitted to the observer. Research directed toward high information content as well as high resolution, particularly utilizing the scanning electron microscope, can help to create the kind of images necessary for subjective information transfer. The secondary electron mode of scanning electron microscope operation produces an image with the important experiential factor of three-dimensional space. We are often aware that the four-dimensional world is not suited to our experiential understanding, but we sometimes forget that a two-dimensional world is equally foreign. A three-dimensional image provides one important parameter that permits the image to approximate experienced vision.

One of the many differences between scanning and transmission electron microscopy is the way in which image information is processed and displayed after leaving the sample. Beside the "normal" intensity-modulated secondary electron image it is possible to display this same information by deflection modulation, or even to plot it graphically. These display
methods make use of the fact that the eye is more sensitive to changes in position than to changes in brightness. As the eye and some recording media are also very sensitive to changes in hue, a method of color modulation has been developed (Fig. 1). This coding method, like deflection modulation, results in a picture lacking the feeling of depth which makes the standard secondary electron image seem so real. However, if the signal is sufficiently noise-free, it allows a larger number of distinguishable signal levels to be displayed on a single picture. It does this while preserving the 1-to-1 correspondence between the position of object and image points, which deflection modulation does not do. Thus, superposition of several color-coded signal modalities in a single image is also possible. In addition to these analytic possibilities, color also provides a powerful way to influence the subjective, intuitive response of the observer. Although the present prototype method is tedious, the means to automate it are readily available through somewhat expensive.

ACKNOWLEDGMENTS

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REFERENCES AND NOTE


The photographic process used to prepare this report does not lend itself to color printing, but illustrative micrographs for this paper can be obtained, in limited quantity, from Donner Laboratory. Color illustrations are also contained in the Scientific Exhibit, "Color and Depth Information as Vehicles for Subjective Information Transfer."
Induction of Psychotic Behavior in Folic Acid Deficient Patients by Ingestion of L-Methionine

Hannes B. Stahelin and H. Saul Winchell

SUMMARY

Three folic-acid-deficient patients, given L-methionine, suffered temporary psychotic disorders. Ingestion of the aminoacid L-methionine in combination with a monoamine oxidase inhibitor induces psychotic behavior in schizophrenic patients. This was first described by Pollin et al. (1), and consequently confirmed by several other studies (2-5). During the course of a study of the effect of L-methionine on the metabolism of $^{14}$C-labeled histidine in folic-acid-deficient patients we observed a striking effect of ingested L-methionine on behavior. The present communication summarizes our findings in three such patients.

CASE REPORTS

Case No. 1

H. B., a 65-year-old white male, was admitted to Highland General Hospital (HGH) with diagnoses of chronic alcoholism, malnutrition, and macrocytic anemia. The bone marrow was megaloblastic. The day following admission he developed hepatic coma which lasted 3 days. Following recovery, he was placed on a normal folic-acid-containing diet. Subsequently, a reticulocyte count of 8% was obtained. Ten days after admission, measurement of his $^{14}$CO$_2$ excretion pattern following an injection of L-histidine (imidazole-2-$^{14}$C) was characteristic of folic acid deficiency (6). With the next 16 hours, the patient received 30 g of L-methionine divided into three equal doses. A few hours following the last dose, he became withdrawn, mute, and apparently disoriented. In reaction to venepuncture he manifested excessive agitation and hostility, but rapidly reverted to his withdrawn passive state. No asterixis was noted. This episode lasted for a day, after which he reverted to his usual aggressive, but oriented, lucid mental state. No history of previous psychotic disorders was obtained.

Case No. 2

J. B., a 42-year-old black male, was admitted to HGH with severe macrocytic anemia, neutropenia, and thrombocytopenia (Hbg 3.8 g %, PCV 12%, RBC 0.8 million/mm$^3$). The bone marrow was megaloblastic. Liver function tests were normal. The history revealed chronic malnutrition and alcoholism. Although a history of previous psychotic episodes could not be documented, the patient's sister reported that a year prior to admission, his mother had died and the patient was subsequently noted to have episodes in which he was withdrawn and appeared to speak irrationally. Diminished appearance of $^{14}$CO$_2$ in the breath of the patient following administration of histidine (imidazole-2-$^{14}$C) 6 days after admission was characteristic of folic acid deficiency. During the study he was lucid and cooperative. He received
two doses of 20 g each on the first day and 20 g more of L-methionine the following morning. Two hours later he became agitated and dis-oriented and began hallucinating. Catatonic withdrawal alternated with agitated hallucinatory periods for 3 days following ingestion of methionine. Oral folic acid therapy (15 mg. per day) was begun on the first day of his psychotic behavior and continued throughout the period of hospitalization. The psychotic episode subsided and the patient was well oriented after 3 days of acute psychosis. Ten days after starting folic acid therapy his PCV was 21%, reticulocyte count 9%, and a repeat breath-\(^{14}\)CO\(_2\) study following administration of L-histidine (imidazole-\(^{14}\)C) was low in amplitude but of normal shape, consistent with folic acid deficiency during erythropoietic response (6). At this time he received two doses of 20 g methionine, each within a 12-h period. Six hr after the second dose he exhibited similar behavioral changes, with agitation and hallucination as described above during the first study. This second episode lasted for several hours. No residual effect was detected 1 day later.

**Case No. 3**

R. T., a 28-year-old white female, was referred to the Donner Clinic with slowly progressive macrocytic anemia related to dermatitis herpetiformis, treated during the previous 1.5 years with sulfoxone sodium. At the time she was first seen she had a Hbg 9.7 g, PCV 28%, RBC 2.28 million/mm\(^3\), serum folic acid level of 0.8 ng/ml. The bone marrow was megaloblastic. The rate of appearance of \(^{14}\)CO\(_2\) in the breath following administration of L-histidine (imidazole-\(^{14}\)C) was at the borderline low normal range. Liver function tests were normal, no history of previous psychotic episodes was obtained. Subsequent to administration of two 10-g doses of L-methionine within a 12-hr interval, the patient became depressed, withdrawn, and irritable, and expressed paranoid feelings towards close members of her family. She withdrew to her room, where she experienced unmotivated outbursts of weeping. When seen by us the following day, her mood was depressed and hostile and she again manifested episodes of unmotivated weeping. Twenty-four hours after cessation of methionine ingestion her behavior returned to its normal pattern. She subsequently recalled the reported episode and commented that it was strange and unique.

**COMMENT**

In the cases reported here, ingestion of L-methionine was rapidly followed by appearance of significant abnormalities of mentation. The total dose of L-methionine administered to these patients was considerably less than that previously used in conjunction with monoamine oxidase (MAO) inhibitors to induce acute relapse in schizophrenic patients (1-5). The temporal coincidence of behavioral alteration with the short loading time with L-methionine leaves little doubt as to the causal relationship between methionine ingestion and the observed abnormal behavior. Furthermore, the symptoms observed in our group are similar to the symptoms described in schizophrenic patients following ingestion of L-methionine in combination with a MAO inhibitor (1-5). Psychotic symptoms were observed by Reynolds (7) in association with folic acid deficiency while studying drug effects in epileptics. Reynolds interpreted the effect of folic acid deficiency as aggravating rather than causing the mental alterations (8).

It is thought that MAO inhibitors act by inhibiting the catabolism of catecholamines and enhancing the formation of methylated "false neurochemical transmitter" in the presence of excessive methionine (4-9). Similarly, the absence of folic acid may inhibit oxidative metabolism of the methyl group of L-methionine.
and somehow enhance the utilization of this methyl group in the synthesis of methylated "false neurochemical transmitters." Indeed, $^{14}$CO$_2$ production is abnormally diminished in folic-acid-deficient human subjects given L-methionine (S-methyl $^{14}$C) (10). The possibility of abnormal transmethylation processes in acute schizophrenia is further supported by the demonstration of an alteration in the metabolism of the methyl group of methionine in acutely schizophrenic patients (11).

In the presently reported group, two patients had no detectable liver dysfunction, but the first case (H. B.) was diagnosed as having alcoholic liver cirrhosis. The mental changes following the ingestion of methionine in this patient could be interpreted as hepatic coma without asterixis. Indeed, one may ask whether hepatic coma is not due in part to excess production of methylated "false neurochemical transmitter" secondary to inhibition of hepatic metabolism of methyl donor substances into other pathways. Although alternative interpretations are available it is noteworthy that ingestion of meat (rich in methyl donors) clinically exacerbates hepatic coma.

Similar abnormalities in mentation were noted in one additional patient in our clinic who had untreated pernicious anemia and a serum folic acid level of 7 ng/ml at the time of methionine ingestion.

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Dosimetry of $\pi$ Mesons Using Silicon Detectors and Plastic Scintillators

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SUMMARY

The dosimetry of both $\pi^+$ and $\pi^-$ beams, with use of semiconductor detectors and plastic scintillators, is described. Depth-dose distributions, isodose distributions, and integral and differential range curves are presented. By use of a time-of-flight system the dosimetric information for a pure pion beam is obtained. The muon and electron contamination reduce the peak-to-plateau ratio. Pion beams of low energy give better depth-dose distributions. Nearly 50% of the dose at the peak of the depth-dose distribution of a $\pi^-$ beam is found to be due to nuclear events.

INTRODUCTION

The dose delivered by a $\pi^-$-meson beam increases slowly with depth, giving rise to a sharp maximum (known as the Bragg peak) near the end of the range. This is like the behavior of other heavy charged particles. When the $\pi^-$ mesons stop in the medium, they are captured by the constituent nuclei. The captured nuclei explode into short-range and heavily ionizing fragments, which enhance the dose at the peak. This may have a radiotherapeutic application because these highly ionizing fragments are effective in decreasing radioresistance of hypoxic cells in a tumor. This idea was informally suggested by a few people, including one of the authors (C. R.), as early as 1952, but detailed calculations by Fowler and Perkins (1) generated heightened interest in the use of $\pi^-$ mesons for radiotherapy. Biophysical experiments have been carried out in this Laboratory over the last 6 years (2-10). Some physical measurements were also made at CERN, Switzerland, and at Brookhaven National Laboratory (11-14). This paper describes the technique used in pion dosimetry and the results obtained by using semiconductor detectors and plastic scintillators.

PRODUCTION OF PION BEAMS

Pions can be produced in a nuclear interaction by any strongly interacting particle if its energy is great enough. They are generally produced by a primary beam of protons. Our experiments at Berkeley are carried out at the 184-inch synchrocyclotron. This machine provides an accelerated beam of 732-MeV protons that strike a 5-cm-thick beryllium target in their outer orbit and produce neutral, positive, and negative pions. The experimental arrangement is shown in Fig. 1. The $\pi^-$ are deflected out of the cyclotron fringe field, and after leaving the cyclotron tank through a window, enter a small quadrupole focusing magnet (meson quad), then travel along a channel (dashed line in Fig. 1) through the main cyclotron shielding (hatched area). The pions then
enter the meson cave, where various arrangements of magnets are used for energy selection and for focusing the beam. A bending magnet is used for momentum selection. The cyclotron produces pions in a range of energies from 0 to about 450 MeV (the upper limit being determined by the energy of the primary proton beam). In the change from a $\pi^-$ beam to a $\pi^+$ beam, all the magnetic fields, including that of the cyclotron, are reversed. The magnetic-lens system remains unchanged for pions of the same energy, regardless of charge.

Neutral pions have a very short lifetime, $\approx 10^{-16}$ sec, and decay into two $\gamma$ rays in the target. The $\gamma$ rays are converted into electron-positron pairs that go mainly in the forward direction. The electrons with the same momentum as pions selected by the bonding magnet constitute electron background in the beam.

Charged pions have a mean life of $2.54 \times 10^{-8}$ sec. Hence some of them decay in flight into muons, and this constitutes the muon background in a pion beam.

The presently available $\pi^-$ beams are too low in intensity for therapeutic applications. (Machines that will produce pion intensities nearly two orders of magnitude higher than the one presently available are under construction at Los Alamos Scientific Laboratory; Vancouver, Canada; Zürich, Switzerland.) The pion beam at the 184-inch synchrocyclotron at Berkeley is the most intense, being $10^6$ particles/sec. In terms of dose rate, it is $\approx 0.5$ rad/min over an area of $3 \times 5$ cm$^2$. With such a beam, however, physical measurements can be carried out quite well.

**EXPERIMENTAL TECHNIQUE**

The characteristics of the pion beam as it passes through an absorbing medium of Lucite, and the energy distribution of $\pi^-$ stars in silicon obtained by using lithium-drifted silicon detectors, have been reported (2). The energies deposited in the detector where pions stop and produce stars have been found to extend beyond 60 MeV. The silicon nucleus with its mass number 28 has $\alpha$-particle structure, and hence the nuclear interaction when $\pi^-$ mesons stop in silicon may not be significantly different from that in other alpha-structure nuclei, such as carbon and oxygen, the main constituents of tissue. The depth-dose distribution, as measured by a silicon detector, is in good agreement with the calculated depth dose based on stopping $\pi^-$ meson interactions in oxygen (6).

The dose deposited in the detector can be measured by integrating the charge liberated in the detector by ionizing radiations such as $x$ and $\gamma$ rays if the leakage current of the detector is annulled by using a balancing circuit (15). With energetic charged particles, however, radiation damage prevents their use at levels above that produced by the leakage current. In the present application these difficulties are alleviated by accepting only the ac pulses, and the pion beam intensity is not high enough to cause significant radiation damage.
The charge liberated in the lithium-drifted silicon detector is directly proportional to the energy deposited by the radiation. A charge-sensitive preamplifier yields a voltage pulse proportional to the energy deposited in the detector, and these pulses are further amplified by a main amplifier. The leakage current of the lithium-drifted detectors is a few microamperes (depending on their thickness), and this current is quite comparable to the current generated in the detector due to pions passing through it. (The total pion intensity seen by the detector is about $5 \times 10^4$/sec.) The detector leakage current is blocked by the ac amplification.

In our previous work, the amplifier output was integrated by using an analog integrator (4). The ac character of the signal necessitated the use of a polarity-clipping circuit (i.e., correcting the negative overshoot) before integration. This system had a linear energy range of 100 to 1. Pion beams are always produced with muon and electron contamination. For a beam of momentum 190 MeV/c, the most probable energy losses—in a 3-mm detector, for example—are 1 MeV by the electrons and 1.2 MeV by the pions; the energy lost by the muons is intermediate between these values (2). There is considerable spread in energy losses due to Landau fluctuations (16, 17). At the end of range the pions stop and produce stars, thereby depositing energies in the detector sometimes exceeding 60 MeV (2). These high energy stars can be observed even with thin lithium-drifted silicon detectors (e.g., 1 mm) because a considerable portion of the star energy is deposited locally. However, in the region where the pion beam enters the medium (usually called the plateau region), the energy deposition in the detector is proportional to the thickness of the detector. The use of a thin detector is advantageous for dose measurements, especially in the pion-stopping region, since this region could be small, for a narrow momentum spread of the primary $\pi^-$ beam. This thin detector necessitates linearity over a wider energy range of the overall system. In our previous work, the linear range was only of the order of 100-fold; we were restricted to using a 3-mm-thick lithium-drifted silicon detector, and it was critical.

The linearity of the system has been improved considerably by changing to digital processing, using a 4096-channel analog-to-digital converter (ADC). The output of the linear-gated amplifier is stretched for compatibility before being fed to the ADC. The ADC translates the signal amplitude to a channel number of a 42-bit binary number. This code is fed to a digital on-line computer, which processes and stores the total number of particles and the total energy. In addition, the computer is also programmed to print out the pulse-height spectrum. The block diagram of the overall system is shown in Fig. 2 and a photograph in Fig. 3. A pulse generator is used to calibrate the system and to check system linearity.

Our primary $\pi^-$ beam is contaminated with 25% electrons and 10% muons. Since the semiconductor detector system is fast and measures the dose due to each particle, it is possible to measure the dose due to pions only, if the muons and electrons are resolved from the pions by using a time-of-flight system and gating the semiconductor detector system with the signal due to pions only.

The time-of-flight system (TOF) measures the time each beam particle takes to travel a fixed distance. Because of space restrictions this length was only 17 feet in our system; increasing the path length would improve the resolution of the system. A plastic scintillation counter is placed at each end of the flight path. In a contaminated pion beam of a given momentum, muons and electrons travel faster than
pions. The velocity spectrum of the particles, as expressed by the time delay between the two scintillation counter pulses, is processed via a time-to-pulse-height converter. The TOF pulse spectra which gate the semiconductor system for the contaminated and pure beam of \( \pi^- \) mesons, and the TOF pion signal, are shown in Fig. 4.

The lithium-drifted silicon detector, housed in an electrically shielded Lucite box, can be remotely positioned in three axes in a water phantom. The data from the semiconductor detector are accumulated in the computer during
the time it takes the monitor scintillator system to accumulate a fixed number of counts. Data are thus collected for each position of the detector in the water phantom.

In addition to the semiconductor detector system, plastic scintillators provide fast coincidence and anticoincidence gating in conjunction with the TOF system, furnishing gating signals for obtaining integral range curves (number-distance curves) and differential range curves (number of particles stopped as a function of absorber thickness). The block diagram of such a setup is shown in Fig. 5. Triple coincidence counts between plastic scintillators 1, 2, and 3 as a function of absorber thickness (normalized by the number of coincidences between 1 and 2) yield the number-versus-distance curve. Normalized triple coincidences between 1, 2, and 3 but with counter 4 in anticoincidence produce the differential range curve. Integral and differential range information for "pure" pion beams was obtained by employing the TOF system.

The cyclotron is pulsed 64 times per second, thus producing 64 coarse groups of pions per second. The mode of cyclotron operation can be controlled so that these groups of pions are spread out over either of two periods. The "short-spill" mode spills the beam over a period of approximately 400 μsec. It is also possible (by use of the auxiliary dee mechanism of the cyclotron) to spread each group of pions over a longer period of 8 to 10 msec. Even in this mode, roughly half of the total beam arrives during the first 400 μsec, producing a spike of beam intensity, and the remainder is stretched over 8 to 10 msec (18). We use the stretched beam and gate off the system during the spike.
to prevent overloading of the electronic system, and thereby avoid the nonlinearity that would occur from saturation.

Because of beam-intensity fluctuations, two plastic scintillators connected to coincidence are used to monitor the beam (see Fig. 2). The various integral and differential counts are collected during a preset number of monitor counts, thereby normalizing all measurements so that beam intensity fluctuations are unimportant.

RESULTS AND DISCUSSION
Dose and Number of Particles as a Function of Depth in Water

The linearity of the semiconductor detector system (including ADC) is checked with a calibrated pulser, and the system is found to be linear from 0.18 to 50 MeV. The energy threshold is set at 0.15 MeV, thereby limiting measurement to energy depositions above this value. The range of linearity can be shifted by adjusting the gain in the main amplifier. This linear range is adequate for the detector thicknesses (1 to 3 mm) used in the investigation reported here.

By measuring the dose in a water container without water in it, the beam used is found to be slightly divergent. The intensity of the beam in air at a position where pions stop and produce stars in water is found to be 15% less than at the point of entrance, due to this divergence in the initial beam.

The depth-dose distribution of $\pi^-$ mesons is measured by using lithium-drifted silicon detectors 1, 2, and 3 mm thick to determine which detector is most suitable for such measurements. The ratios of the dose at the peak to that at the entrance for the three detectors are found to be the same within the experimental accuracy. In addition, the energy deposited per $\pi^-$ at the peak region is also found to be proportional to the thickness of the detector. These results indicate that the pion stars deposit energy over a finite range comparable to the thicknesses of the detectors used.

For the rest of the measurements, a lithium-drifted silicon detector 2 mm thick is used. As mentioned earlier, the computer printout gives the total number of particles passing through the detector as well as the total energy deposited by them. When the semiconductor detector system is gated by the TOF pion signal, data for essentially pure $\pi$ mesons (without the muon and electron contamination) are obtained. The plots of the total number of particles passing through the detector and the total energy deposited by them as a function of depth in absorber (water) give the integral range (also called number-distance) and the depth dose curves respectively. Figure 6 shows such curves, both for contaminated and for pure $\pi$ meson beams of energy 65 MeV for the semiconductor detector.

Measurements are taken for a $\pi^+$ beam for comparison with the $\pi^-$ results. Positive pions with the same momentum as $\pi^-$ mesons can be produced by reversing the polarity of the whole magnetic system, including the field of the cyclotron.

The characteristic difference in interaction between $\pi^+$ and $\pi^-$ mesons is at the ends of their ranges. When the $\pi^+$ meson comes to rest in a medium, the Coulomb repulsion keeps it from being captured in an atomic orbit. Instead, it decays into a muon with an energy of 4 MeV, which then decays into a positron with an energy distribution peaking around 30 MeV. Hence at the end of the range, the $\pi^+$ does not add much to the dose except for a small contribution due to the muon and positron.
The electron contamination in a $\pi^+$-meson beam is only around 10%, compared with 25% for a $\pi^-$-meson beam, whereas the muon contamination is the same for both the beams. If depth-dose distributions of pure beams of $\pi^+$ and $\pi^-$ mesons, obtained by using the TOF gating of the semiconductor detector system, are compared, the difference can be attributed to the star events from $\pi^-$ capture, except for the small contribution due to muons and positrons resulting from the $\pi^+$ decay.

In order to evaluate the dose contribution due to $\pi^-$ stars, the depth-dose distributions of pure $\pi^-$ and $\pi^+$-meson beams of energy 65 MeV in water are measured; they are shown in Fig. 7.

**Differential and Integral Dose Distribution**

The differential and integral dose distributions can be computed from the measured pulse-height distribution at any point. The differential dose distribution gives the distribution of energy depositions in the detector, whereas the integral dose distribution gives the total energy deposited in depositions greater than a particular energy. The differential and normalized integral dose distributions at the peak of the $\pi^-$ depth-dose curve are shown in Fig. 8. The energy of a pion that has a range of 2 mm of silicon, the detector thickness used, is about 8 MeV. Hence energy depositions greater than 8 MeV are due to star events. As can be seen from the differential dose distribution, the area under the curve over an energy range of 0 to 8 MeV approximates the area under the curve above 8 MeV. Hence the dose contribution due to star events is about half of the total dose at the peak region of a depth-dose distribution for a $\pi^-$ contaminated beam. The peak at 50 MeV in differential dose distribution is a saturation effect; any event depositing energies greater than 50 MeV is counted as 50 MeV.

Similarly, from the plot of the normalized integral dose distribution it is seen that the fraction of the dose due to energy depositions...
The normalized integral dose distribution for pion beams at various depths are shown in Fig. 9. The two curves for the $\pi^-$ beam corresponding to the peak and halfway down the falling portion (trailing edge) of the depth-dose distribution are similar, thereby showing that the ionization density at the peak and thereafter is about the same. On the other hand, the curve corresponding to the point halfway up the rising portion (leading edge) of the depth-dose curve falls below the other two curves. The ionization density at this point is considerably less. This is expected because the $\pi^-$ mesons making stars at and beyond the peak of the depth-dose curve have to pass this point and hence deposit dose of lower ionization density. The dose fractions due to the star events in pure and contaminated $\pi^-$ beam are 55 and 45% respectively. In the $\pi^+$ beam at the peak of the depth-dose curve, the dose fraction greater than 10 MeV is very small. The contribution above 8 MeV is due to the additional energy deposition from $\mu^+$ and $e^+$ arising from the decay of $\pi^+$ near the end of the range.
**Isodose Contours**

Isodose contours give a much clearer picture of the spatial dose distribution. Such curves can be constructed from a series of beam profiles taken in water at various depths. Figures 10 through 13 show such isodose contours for contaminated as well as for pure $\pi^+$ and $\pi^-$ beams of energy 65 MeV in water. The distortion of the isodose contours is due to a slight nonuniformity in particle momentum laterally across the beam.

**Depth-Dose Distribution of 90-MeV and 65-MeV $\pi^-$ Beams in Lucite**

To minimize the effect of the divergence of the beam on the depth dose distribution, the detector position was fixed and the thickness of the Lucite absorber varied. With the same setup, the data for integral and differential range curves when plastic scintillators are used were also obtained and are presented in the next section. Figure 14 shows the depth-dose distribution of contaminated as well as pure $\pi^-$ beams of energies 65 and 90 MeV. It can be
seen that for the pure 90-MeV $\pi^-$ beam, the dose at the peak (right) is not significantly different from that for the contaminated beam, whereas at 65 MeV the peak for the pure beam is considerably higher than for the contaminated one. This is because of less contamination in the 90-MeV $\pi^-$ beam. The lower value of the peak-to-plateau ratio for the high energy beam is mainly due to loss of particles by nuclear attenuation and multiple scattering before reaching the end of the range.

CONCLUSIONS

This series of experiments demonstrates the complexities in providing adequate dosimetric information for pion beams. In addition to the variation of dose with depth, there are significant differences in the quality of dose. Contamination not only reduces the peak-to-plateau ratio, but also increases the relative contribution of dose due to low-LET radiation. Low-energy $\pi^-$ beams give higher peak-to-plateau ratios.

Measurements of Differential and Integral Range Curves with Plastic Scintillators

Differential and integral range curves were obtained with the scintillator counter array. The block diagram of the scintillation counter systems for obtaining these curves simultaneously with the semiconductor detector system is shown in Fig. 5. The integral range curves obtained with both the systems are found to be the same. The differential range curves—i.e., the number of particles stopping in Counter 3 as a function of Lucite absorber thickness for pure and contaminated 90-MeV and 65-MeV $\pi^-$ beams—are shown in Fig. 15. Such curves are very useful in determining the depth at which most of the particles stop.
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REFERENCES AND NOTES


This paper has been submitted to Physics in Medicine and Biology.
RBE and OER at Various Points on the Modified Depth Dose Distribution of 910-MeV Helium Ion Beam Using Cultured Cells (T1)

Mudundi R. Raju, Madvanath Gnanapurani, Udipi Madhvanath and John T. Lyman

SUMMARY

The relative biological effectiveness (RBE) and oxygen enhancement ratio (OER) at different points on the modified depth-dose distribution of a 910-MeV helium ion beam is measured by using human kidney cells in culture (T-1). The results indicate that the RBE at the broad peak region is slightly higher than the effect at the beam entrance (plateau), and the OER is found to be significantly reduced.

The 910-MeV helium-ion beam from the 184-inch synchrocyclotron at Berkeley has long been used mainly for pituitary irradiations. It is of interest to find out how a small fraction of dose due to high-LET components at the broadened Bragg peak region would affect the radiobiological properties of therapeutic interest such as relative biological effectiveness (RBE) and oxygen enhancement ratio (OER). Such measurements will be helpful in assessing the therapeutic capabilities of this radiation. With this in view, preliminary experiments were performed with human kidney cells in culture.

The experimental techniques developed by Barendsen (1) and Todd (2) were used in this investigation. Feeder cells (5 x 10^4/dish) exposed to 4000 rads of X rays were plated in 35-mm Petri dishes and the dishes were incubated overnight. Cells in early logarithmic growth phase in appropriate numbers were plated in the dishes with feeder cells so that surviving colonies would be about 100 on each dish. The dishes were placed in the incubator at 37°C for 4 to 7 hr, before exposure to radiation.

A "sample loading wheel" as described by Todd (2) was used. The medium was removed from the dishes before they were mounted in the wheel. Air or nitrogen saturated with water vapor was admitted into the wheel and circulated through the dishes during exposure. In the case of nitrogen, the cells were exposed to the nitrogen atmosphere for at least 5 min before exposure to radiation.

The Bragg peaks of monoenergetic charged particle beams are narrow when compared with typical tumor sizes. The technique of modifying the depth-dose distribution of monoenergetic charged particles to uniformly irradiate the treatment volume is usually done by using ridge filters (3, 4). The depth-dose distribution of a 910-MeV helium-ion beam was modified by using a ridge filter so that the peak of the depth-dose distribution is broad enough to
Fig. 1. Modified depth-dose distribution of a 910-MeV helium-ion beam used with a ridge filter, showing the three exposure positions.

DBL 703-5627

cover about 4 cm of Lucite. Such a distribution is shown in Fig. 1. Dishes containing cells were exposed to different doses of radiation at three points marked 1, 2, 3 on the depth-dose distribution. Three dishes containing cells were exposed for each dose point. Twelve to 15 days after exposure, the colonies were fixed in Bouin's fluid stained with Harris's hemotoxylin. All the visible colonies were counted and the percentage survival calculated.

Figure 2 gives the survival curves obtained for the three positions on the depth-dose distribution mentioned above. Biological effectiveness when compared with that of the plateau position (as marked 1 in Fig. 1) and OER were calculated in the surviving-fraction region of about 10%.

The biological effect at the plateau is very similar to that of conventional radiation. The biological effectiveness at positions 2 and 3 compared with position 1 are 1.1 and 1.3 respectively. The OER values at positions 1, 2, and 3 are 2.5, 2, and 1.8 respectively. These preliminary results indicate that there is a significant reduction in OER in the broad peak region. If the exposure were made with two opposing fields one could obtain an average OER of 2 over a 4-cm-wide region.

Two more experiments with T-1 cells and one experiment using Chinese hamster cells gave similar results. Further work is in progress.

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The author (M. R. Raju) is grateful to Dr. G. W. Barendsen for introducing him to tissue culture work. We wish to thank Mrs. Catharine D. Boerke for her suggestions in maintaining a tissue culture laboratory.

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RBE and OER of $\pi^-$ Mesons for Induction of Heteroallelic Reversion in Diploid Yeast

Mudundi R. Raju, Madvanath Gnanapurani, Udipi Madhvanath, Benjamin Stackler and Robert K. Mortimer

SUMMARY

Induction of heteroallelic reversion in diploid yeast is one of the most radiosensitive systems known. Arginine reversion in a diploid strain BZ34 of Saccharomyces cerevisiae has been used to measure the OER for $^{60}$Co $\gamma$ rays and $\pi^-$ mesons. The values obtained are 2.4 and 1.8 respectively. The response of this system is found to be the same for $^{60}$Co $\gamma$ rays at dose rates of 40 rads/hr and 40 rads/min.

Radio-sensitive systems are needed for the evaluation of $\pi^-$ mesons for radiotherapy with the presently available low-intensity pion beams. It is known that sublethal doses of X rays result in a large increase in reversion to wild type in diploid yeasts that are heteroallelic for mutations at a given locus (1, 2). In this work, an attempt has been made to measure the oxygen enhancement ratio (OER) for induction of reversion by $^{60}$Co $\gamma$ rays and $\pi^-$ mesons in such a heteroallelic strain of diploid yeast. The relative biological effectiveness (RBE) of $\pi^-$ mesons compared with $^{60}$Co $\gamma$ rays is also measured.

EXPERIMENTAL PROCEDURE

A diploid strain BZ34 of Saccharomyces cerevisiae described earlier by Fogel and Mortimer (3) was used in this study. Its genotype is

\[
\begin{align*}
\alpha &+ \text{arg} 4-4 + \text{thr} 1 \\
\text{pet} 1 &+ \text{arg} 4-17 \\
\text{trp} 5-48 &+ \text{ura} 3 \\
\text{leu} 1-12 &+ \text{try} 5-48 \\
\text{his} 5-2 &+ \text{met} 1 \\
\text{lys} 1-1 &+ \text{ade} 2-1
\end{align*}
\]

arg 4-4 and arg 4-17 are a noncomplementing combination of mutant alleles of the arginosuccinase locus. Reversion for arginine requirement was studied by omitting arginine from synthetic complete medium. This strain was chosen because the two arg 4 alleles are located far apart in the locus, hence small doses of radiation can be detected by an increase in reversion frequency.

For $^{60}$Co irradiation a 150-Ci source was used to deliver a dose rate of 40 rads/hr. In addition, a 1500-Ci source was used at a dose rate of 40 rads/min.

For $\pi^-$-meson irradiation, the 184-inch synchrocyclotron was used. The dosimetry and details of the irradiation facility are described elsewhere (4). The dose rate was 30 rads/hr at the peak of the depth-dose distribution of the beam.

The cells were cultured and grown in 30 ml of liquid YEPD (5). After 2 days of incubation at 30°C, the cells were centrifuged, washed, and resuspended in water to a concentration of...
It was possible to position six of these samples within the peak region. The sample disks were mounted in a 35-mm petri dish which in turn was fitted into a Lucite box cooled by circulating cold water. Precooled moist air or nitrogen was passed through the box during exposure. The temperature of the samples during irradiation was about 5°C. Since, in some cases, irradiation time was long, a low temperature was necessary to avoid any increase in the frequency of arginine revertants due to storage in nonnutrient medium (6). Figure 1 shows the number of arginine revertants induced during storage at room temperature and at 5°C. Duplicate samples were exposed for each dose point. A control set of samples in similar boxes was maintained at the same temperature for both aerobic and anoxic conditions.

After exposure the cells were resuspended in sterile water and 0.3 ml/plate was plated on arginineless plates. Thus, the number of treated cells/plate was \(3 \times 10^6\). Serial dilution was made for plating on YEPD plates to assay survival. The doses used in this investigation were sublethal. Five arginineless and five YEPD plates were used for each sample. The colonies were counted on the plates after 3 days of incubation at 30°C. A recount of the AR plates was made on the sixth day. The ratio of the average number of colonies on the AR plates to those on the YEPD plates multiplied by the appropriate dilution factor is the frequency of mutants per survivor for a given dose.

**RESULTS**

Figure 2 is a plot of the frequency of revertants/survivor vs dose for acute and chronic exposure of \(^{60}\)Co \(\gamma\) rays under aerobic and anoxic conditions. The ratio of the slopes of the lines is an index of the oxygen effect. The results indicate that the mutation-induction efficiencies for dose rates of 40 rad/hr and 40 rad/min are about the same. The OER for \(^{60}\)Co \(\gamma\) rays for induction of this effect is 2.4.

In Fig. 3 are presented the results obtained (from two experiments) for irradiation with pions. They indicate a value of 1.8 for the oxygen effect. An RBE value (aerobic) of 1.5 for mesons relative to \(^{60}\)Co \(\gamma\) rays is obtained by comparing the slopes in Figs. 2 and 3. Thus, a significant reduction in OER is obtained in spite of the contamination of \(\mu\) mesons and electrons (35%, expressed as number of particles) in the beam. These RBE and OER values may also be applicable for acute exposure to \(\pi^-\) mesons.
Fig. 2. Frequency of arginine-independent revertants as a function of 60Co γ-ray dose in atmospheres of air and nitrogen, at dose rates of 40 rads/hr and 40 rads/min.

Fig. 3. Frequency of arginine-independent revertants as a function of π^- meson dose in atmospheres of air and nitrogen.

The response of this system to heavy ions is being studied.

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Graduate Students

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<tr>
<td>Richard P. Bird</td>
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<td>Sylvia Spengler</td>
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<td>Benjamin Stackler</td>
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Engineering and Technical Support Staff

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<tr>
<td>Robert C. Aker</td>
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<td>Dorothy E. Denney</td>
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<td>John A. Despotakis</td>
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<tr>
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<tr>
<td>John P. Flambard</td>
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<td>John R. Gurule</td>
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</table>
Engineering and Technical Support Staff
(Continued)

Edward A. Heilstad
David A. Hoopes
Victor P. Jensen
Edward J. Lampo
Ellis H. Myers
Karen A. Peterson
Lydia K. Romero
Gladys V. Short
Marjory H. Simpson
John M. Sweeney
Frank T. Upham
Alfred A. Windsor

Administrative and Office Staff

Igor R. Blake
Mary M. Bryan
Janice C. DeMoor
Marijan F. Edelen
Fern V. Forsberg
Joan E. Fox
Betty M. Gale
Maris Gavzy
Dorothy B. Hedquist
Jessica Inskeep
Marcia D. Jackson
Maxine Mangels
Judith S. Manning
Dorothy D. Meck
Pamela A. Mott
Shirley C. Peterson
Michael A. Sauceda
Ruth T. Showalter
Dorothy S. Sprague
Grace V. Walpole