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Semiannual Report to
THE ATOMIC ENERGY COMMISSION

SEPTEMBER 1963

LEON O. JACOBSON, M.D.
Editor

MARGOT DOYLE, Ph.D.
Associate Editor

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LEUKEMIA AMONG CHILDREN IN A SUBURBAN COMMUNITY[*]

By

C. W. Heath, Jr.[†] and R. J. Hasterlik

Between the autumn of 1957 and the summer of 1960 eight cases of leukemia were diagnosed among children living in the town of Niles, a suburb of Chicago in Cook County, Illinois. These cases represented for this town a significantly increased annual incidence of leukemia among children, 21.3 cases per 100,000 in Niles compared with 4.6 cases per 100,000 in the remainder of Cook County (Table 1). The clinical and epidemiologic features of this unusual concentration of leukemia cases are presented in this report.

Each of the eight children with leukemia in Niles lived in the same residential neighborhood, an area which corresponds with the Roman Catholic parish of St. John Brebeuf. Seven of the eight children were from Roman Catholic families, and each of these seven children attended or had older siblings who attended the single parochial grade school in the parish, the St. John Brebeuf Elementary School. The eight cases occurred in a particular time pattern, and they were accompanied by the parallel appearance of a "rheumatic-like" illness among other children attending the St. John Brebeuf School.

No common factor was found to provide an explanation for the occurrence of these eight cases of leukemia. Their epidemiologic characteristics, however, suggest a relationship to infectious processes, in addition to providing material of general interest for the study of leukemia.

NILES AND ST. JOHN BREBEUF PARISH

Niles is a suburban town adjoining northern Chicago in Cook County, Illinois (Fig. 1). The St. John Brebeuf Parish is located in the northern part of Niles and covers an area between 1 and 2 square miles in size (Figs. 1 and 2a). The parish area is entirely residential, and its population, estimated at 17,870 in May 1961,[1] constitutes the major part of the entire population of Niles.

During the decade 1950-1960 Niles, together with most surrounding communities, experienced a striking growth in population. The town expanded from 3,587 in 1950 to 20,393 in 1960,[‡] and most of this growth took place in the parish area. Of all families resident in the parish area in May 1961 about 80 per cent did not move to Niles until 1955 or later.[1]

As a result of this recent expansion, the parish community is newly developed. Housing consists primarily of single-family homes of modern and uniform design, the majority of which have been built since 1955.[1] The community fuses on the south and southwest with older neighborhoods of Chicago and Park Ridge, Illinois, and on the southeast with a smaller and older

Table 1

INCIDENCE OF LEUKEMIA AMONG WHITE CHILDREN UNDER AGE 15 IN
NILES, ILLINOIS, AND VICINITY, 1956 - 1960

Area	Population under age 15 (1960 census)	Cases of leukemia in children under age 15,[*] 1956 - 1960			Average annual rate per 100,000
		Recorded deaths	Physician reports[†]	Total	
Niles	7,076	2[‡]	6	8[‡]	21.3
Neighboring towns	44,075	10	3	13	5.6
Des Plaines	11,952	3	-	3	4.7
Lincolnwood	3,654	1	-	1	5.2
Morton Grove	7,995	2	2	4	9.4
Skokie	20,474	4	1	5	4.6
Cook County, Illinois, exclusive of Niles	1,152,695	241	45	286	4.6

[*]Cook County deaths recorded by age at death. All other cases recorded by age at diagnosis.

[†]From survey conducted April 1961. Includes only cases diagnosed prior to January 1, 1961, in which patients survived beyond that date.

[‡]Excludes one case in which onset occurred prior to residence in Niles (see text).
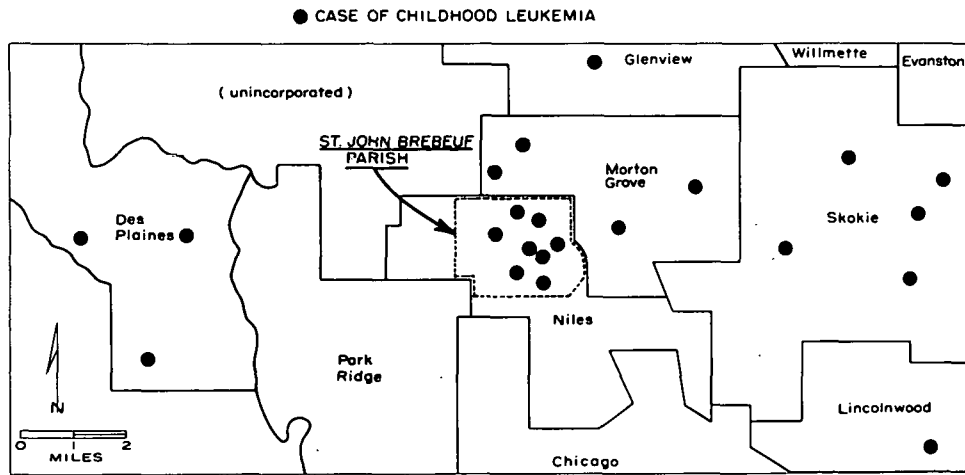
Figure 1. Leukemia among children under age 15, Niles, Illinois, and vicinity, 1956-1960.

residential section of Niles (Figs. 1 and 2a).

About 60 per cent of all families in the St. John Brebeuf Parish area are Roman Catholic and attend the St. John Brebeuf Church. Of all families with children who attend elementary school, a similar proportion is Roman Catholic, and of these Roman Catholic families about 70 per cent use the St. John Brebeuf School. Catholic and non-Catholic families live intermingled throughout the parish area.

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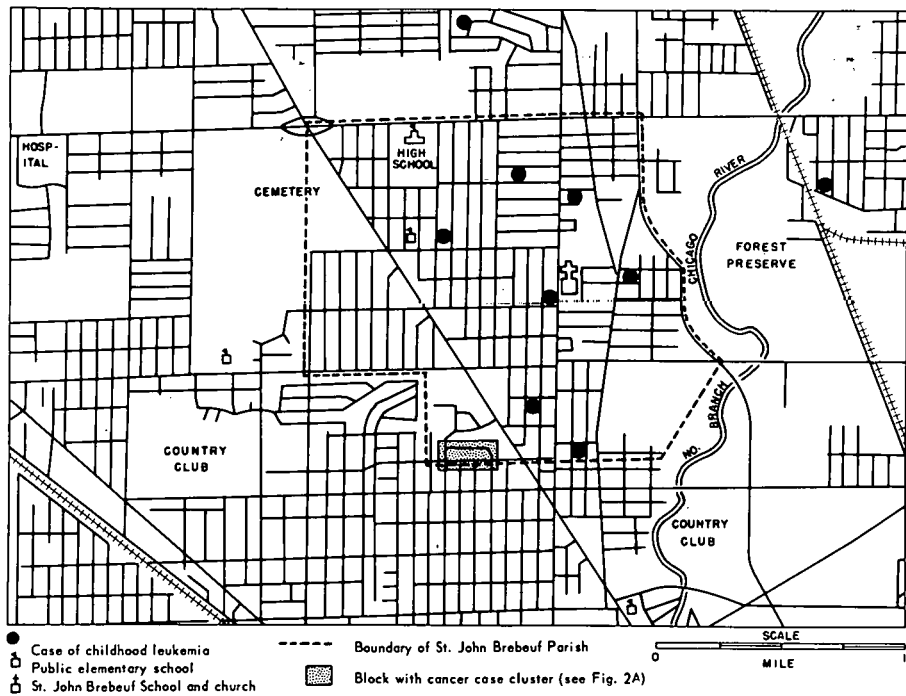
Figure 2a. Leukemia among children, St. John Brebeuf Parish in Niles, Illinois, 1956-1960.
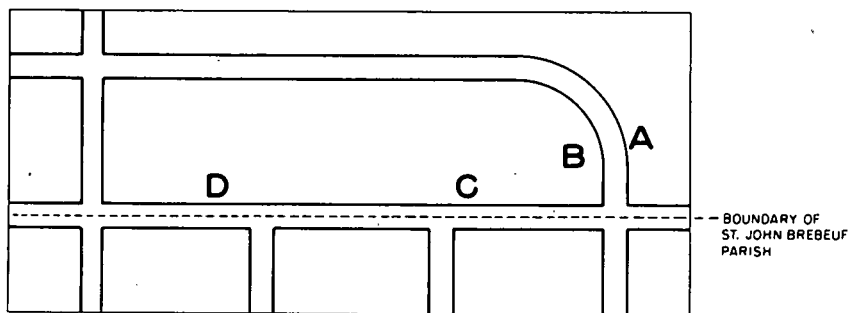
CASE	AGE	SEX	DIAGNOSIS
A	9	M	HODGKIN'S DISEASE
B	40	M	ACUTE LEUKEMIA
C	59	F	ACUTE LEUKEMIA
D	11	M	EWING'S SARCOMA

Figure 2b. Leukemia among adults and cancer among children in families living in a single block area of St. John Brebeuf Parish in Niles, Illinois, 1956-1960. Case A is a nine year old boy with a diagnosis of Hodgkin's disease. Case B is a forty year old man with a diagnosis of acute leukemia. Case C is a fifty-nine year old woman with a diagnosis of acute leukemia. Case D is an eleven year old boy with a diagnosis of Ewing's sarcoma.

The entire population is white, with a wide range of national origins. The majority of families moved to Niles from the vicinity of northern Chicago. Except for differences in religious affiliation and school attendance, families in the parish area are generally similar in regard to

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length of residence in Niles, educational and occupational status, and general medical care.[1]

Elementary schools serving the parish area include the St. John Brebeuf Elementary School, three public grade schools and two public junior high schools. Four of the public schools are located in Niles (Fig. 2a) and one in the adjoining town of Skokie. All five public schools draw pupils from neighboring communities as well as from the parish area. Enrollment in these five schools during the school year 1960-1961 totaled 2,723 children from kindergarten through eighth grade. Classes in the public schools averaged 20 to 30 pupils each.

The St. John Brebeuf School enrolls pupils exclusively from Roman Catholic families living in the parish area. In 1960-1961 it had an enrollment of 1,430, twice as large as the community's largest public elementary school. Individual classes averaged 50 pupils each and included grades 1 through 8. The school was first opened in the fall of 1955 with 384 pupils and 11 classrooms; it was expanded in 1957 to its present size of 29 classrooms.

The St. John Brebeuf School is centrally located in the parish area (Fig. 2a). It consists of a modern, one-story building with classrooms arranged around a central open court, and it shares a common entrance hall with the adjoining parish church. Through religious services, school sessions and other organized activities the church and school function as a community center, serving Roman Catholic families of the parish exclusively. In providing such consolidated community activities the St. John Brebeuf School and Church contrast sharply with other churches and schools in the parish area. As a result, Roman Catholic families, particularly those with children who attend the St. John Brebeuf School, receive greater opportunity for interpersonal contact than do their non-Catholic neighbors.

METHODS

In examining the occurrence of leukemia among children in Niles particular attention was given to establishing as completely as possible an accurate list of cases among residents both of Niles and of adjacent towns. With the assistance of the Illinois Department of Public Health photocopies of death certificates were obtained for all residents of these towns who had died of leukemia while under the age of 20 between 1956 and 1960. This set of cases was supplemented through a questionnaire survey in April 1961 of selected physicians in the greater Chicago area, all members of a hematologic association. In this survey data were gathered concerning patients with leukemia under the age of 20 at the time of diagnosis who were currently being treated or who had died since January 1, 1961.

From this information lists were compiled of cases of leukemia which occurred in Niles and vicinity between 1956 and 1960 among children under the age of 15 at the time of diagnosis (Table 1). To provide further comparative data tabulations were also obtained for all persons who died of leukemia in all of Cook County, Illinois, during the years 1956-1960.

The eight cases of leukemia among children in Niles were each reviewed in detail. Medical and family information was gathered from the records of physicians and hospitals and in home interviews with each family. Diagnostic specimens of bone marrow and peripheral blood were obtained in six of the eight cases and were reviewed by Mila I. Pierce, M.D., Department of Pediatrics, University of Chicago.

A survey of the population living within the St. John Brebeuf Parish area was conducted in May 1961, with the assistance of the Cook County Department of Public Health and the Statistics Section of the Epidemiology Branch, Communicable Disease Center.[1] In this survey information

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was gathered concerning various population characteristics according to family church affiliation and school attendance. Data were also obtained regarding the recent occurrence of common childhood illness among these different family groups. The survey procedure involved the random selection of 37 pairs of adjacent blocks from the parish area total of 184 occupied blocks. The occupants of approximately one fourth of all dwelling units within each of these block pairs were selected at random for interview. Of 529 occupied dwelling units thus sampled, interviews were completed in 479, yielding an approximate 10 per cent random sample of the total parish area population.

Childhood mortality in Niles from causes other than leukemia, in particular from other forms of cancer, was examined through photocopies of death certificates obtained for all residents of Niles and vicinity who died under the age of 15 between 1956 and 1960. Similar data were gathered for cases of leukemia among adults. Additional information concerning non-fatal cases of cancer, as well as concerning the occurrence of "rheumatic-like" illness among children in Niles, was collected directly from public and parochial school authorities serving the St. John Brebeuf Parish area.

Several further studies were also conducted. Through the Cook County Department of Public Health information was gathered from veterinarians in Niles and in adjacent towns regarding the local prevalence of leukemia among animals. With the assistance of the Epidemiology Branch, National Cancer Institute, levels of background radiation were measured in the buildings of the St. John Brebeuf School and Church and in the homes of several of the patients with leukemia. Finally, specimens of venous blood were obtained from all members of each family in Niles in which a child had leukemia. These specimens were stored in anticipation of future research interests.

<div align="center">LEUKEMIA AMONG CHILDREN</div>

The concentration of cases of leukemia among children in the St. John Brebeuf Parish area first came to medical attention in the early spring of 1961 when the death of four children from leukemia during the first three months of that year aroused community concern. Subsequently, a total of eight cases was found to have been diagnosed since 1957, seven from Roman Catholic families whose children attended the St. John Brebeuf School (Table 2). The clustering of these eight cases within the parish community is apparent in Figure 1, in contrast to the more scattered occurrence of cases in neighboring towns.

Attack rates. Table 1 compares the incidence of leukemia in children for Niles with similar rates both for neighboring towns and for all of Cook County, Illinois, exclusive of Niles. Only the rate for Niles is significantly different (P = 0.007) from the rate for Cook County. These rates are not strictly equivalent to standard rates of incidence for leukemia in children.[*] They are, however, reasonably comparable among themselves, although the individual investigation of cases outside of Niles was not as thorough as that within Niles.

Table 2 shows the incidence of leukemia in children among different family groups within the St. John Brebeuf Parish area.[†] This table compares all families with children under the age

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[*] The rates in Table 1 are based both upon deaths recorded during the five year period, 1956-1960, and upon patients living during the first four months of 1961.

[†] The rates in Table 2 are based upon maximum estimates of time and population. During the years in which the eight cases of leukemia occurred, the population of the parish area was

Table 2

INCIDENCE OF LEUKEMIA AMONG CHILDREN UNDER AGE 15[*] IN ST. JOHN BREBEUF PARISH (ST. JB)
IN NILES, ILLINOIS, 1956 - 1960

Family groups	Families with children attending elementary school[†]									Families with children not attending elementary school[†]			Total families		
	Attend St. JB School			Not attend St. JB School			Pop.	Total cases	Rate	Pop.	Cases	Rate	Pop.	Cases	Rate
	Pop.	Cases	Rate	Pop.	Cases	Rate									
Attend St. JB Church	2,340	7	59.8	940	-	-	3,280	7	42.7	810	-	-	4,090	7	34.2
Not attend St. JB Church	-	-	-	1,880	1	10.6	1,880	1	10.6	930	-	-	2,810	1	7.1
Total families	2,340	7	59.8	2,820	1	7.1	5,160	8	31.0	1,740	-	-	6,900	8	23.2

[*]Rates per 100,000 based on populations under age 15 during spring 1961 [1].

[†]Includes kindergarten through eighth grade.

of 15 according to church and elementary school attendance, and it demonstrates the marked concentration of cases among those families who use the St. John Brebeuf School. No cases were observed either among Roman Catholic families with children attending elementary schools other than the St. John Brebeuf School, or among families with children attending no elementary school at all. The rate of incidence for families who use the St. John Brebeuf School (59.8 cases per 100,000) is significantly increased (P = 0.04) over the rate for all other families, Catholic or non-Catholic, who use elementary schools other than the St. John Brebeuf School (7.1 cases per 100,000).

Diagnosis. The diagnosis of leukemia was established in each of the eight cases by hematologists consulting at the time of illness. These diagnoses were confirmed during the present investigation by review of clinical records in all eight cases, and in six of the eight by microscopic examination of bone marrow preparations with companion smears of peripheral blood. In Cases 3 and 7 satisfactory specimens of blood and marrow were no longer available, although a biopsy specimen of lymphosarcoma tissue was examined in Case 3. With the exception of these two cases, the diagnoses listed in Table 3 are the diagnoses made on review at the University of Chicago. Since the various specimens differed technically, a morphologic comparison among them was not practical. However, of the six reviewed, five were described as "stem cell" leukemia and one as "lymphoblastic" leukemia. The remaining two cases were designated at the time of initial diagnosis as "acute lymphocytic" leukemia.

CASE REPORTS

The following case synopses present specific clinical and epidemiologic data for each of the eight cases of leukemia. Selected characteristics of these cases and of the families in which they occurred are summarized in Tables 3 and 4.

Case 1. This 3-year-old white boy (J.R.) became ill late in September 1957, with fever, calf pain and tender bilateral cervical lymphadenopathy. He became pale, had a cough and a persistent fever and was hospitalized in October 1957. Hematologic studies showed a hemoglobin of 3.0 gm per cent, a white blood cell count of 2,100 per cu mm with a predominance of lymphocytes, and almost complete replacement of a tibial bone marrow specimen with "stem cells." An infiltrate at the base of the left lung was demonstrated on a chest roentgenogram. The child was given blood transfusions and was treated with steroids and Achromycin.® He showed little improvement and died on November 3, 1957, six weeks after onset.* Autopsy was not performed.

Comment: This child's health had previously been excellent. His birth was normal, although his mother had "flu" during the second trimester of pregnancy. He had never been exposed to x-rays. There was no history of cancer in the family, and neither parents nor siblings were sick at the time of the patient's onset.

His family had moved to Niles from Chicago three years before, and they attended the St. John Brebeuf Church regularly. The patient was the fourth of five children (three girls and two

somewhat smaller than indicated by the figures listed. The 1961 population estimates, however, are suitable for comparing the relative incidence of leukemia among different family groups in the parish since these groups all had similar rates of growth during this period of population growth.[1]

*The term "onset" of disease is used repeatedly throughout this report and in each instance refers to the initial appearance of clinical signs or symptoms associated with the illness later diagnosed. In most cases the month of "onset" could be determined with reasonable certainty.

Table 3

CLINICAL CHARACTERISTICS OF EIGHT CASES OF LEUKEMIA AMONG CHILDREN OF
ST. JOHN BREBEUF PARISH IN NILES, ILLINOIS, 1956 - 1960

Case no.	Initials	Sex and age (yr) at onset	Month and year of onset	Date of death	Duration of illness (mo)	Features of clinical course	Diagnosis
1	J.R.	M, 3	9/57	11/3/57	1-1/2	Fever, leg pain, cervical adenopathy anemia, pneumonitis	Stem cell leukemia
2	J.B.	F, 4-1/2	10/57	-		Fever, anemia, leg pain	Stem cell leukemia
3	K.S.	F, 10	4/58(?)	1/11/61	33	Maxillary sinus lymphosarcoma, fever, anemia, splenomegaly	Lymphosarcoma of right maxillary sinus, acute lymphocytic leukemia
4	L.B.	F, 9	11/59	2/23/61	15	Fever, leg pain, pneumonitis, thrombocytopenia, gastrointestinal hemorrhage	Stem cell leukemia
5	K.E.	F, 12	12/59	10/28/60	10	Fever, cervical and mediastinal adenopathy, uremia, hepatosplenomegaly, thrombocytopenia, gastrointestinal hemorrhage	Lymphoblastic leukemia
6	M.R.	F, 3	6/60	-		Fever, anemia, thrombocytopenia, involvement of spinal cord, hepatosplenomegaly	Stem cell leukemia
7	E.S.	F, 13-1/2	8/60	1/8/61	6	Fever, anemia, shoulder pain, hepatosplenomegaly, gastrointestinal hemorrhage	Acute lymphocytic leukemia
8	T.S.	F, 6-1/2	8/60	3/14/61	7	Fever, cervical adenopathy, anemia thrombocytopenia, hepatosplenomegaly, gastrointestinal hemorrhage	Stem cell leukemia

Table 4

FAMILY CHARACTERISTICS OF EIGHT CASES OF LEUKEMIA AMONG CHILDREN OF
ST. JOHN BREBEUF PARISH (ST. JB) IN NILES, ILLINOIS, 1956 - 1960

Case no.	Initials	At time of onset			Church attendance	Occupation of father	Principal national origins	
		Residence in Niles (yr)	School attendance				Mother	Father
			Case	Siblings				
1	J.R.	3	-	St. JB	St. JB	Truck driver	Lithuania	Germany
2	J.B.	1-1/2	-	St. JB	St. JB	Pipe fitter	Poland	Sweden
3	K.S.	3	Public	Public	Lutheran	Orthodontic technician	Germany	Germany
4	L.B.	1	St. JB	St. JB	St. JB	Truck driver	Poland	Poland
5	K.E.	10	St. JB	St. JB	St. JB	Insurance business	France	Scotland
6	M.R.	2-1/2	-	St. JB	St. JB	Truck driver	Italy	Italy
7	E.S.	5	St. JB	St. JB	St. JB	Fluorescent light manufacture	Poland	Poland
8	T.S.	7	-	St. JB	St. JB	House painter	Germany	Ireland

boys). At the time of his illness two older sisters were attending the St. John Brebeuf School, one in the second grade and one in the fourth. In the eldest sister, as will be discussed later, a "rheumatic-like" illness developed during the fall of 1959.

Case 2. In October 1957 this 4-1/2-year-old white girl (J.B.) had fever, diarrhea and cough. A scheduled tonsillectomy was postponed because of continued, intermittent fever. In January 1958 she became acutely ill with a temperature of 103°F and pain in her legs and back. She was noted to be pale and easily fatigued and was admitted to a hospital in February 1958 because of epigastric pain and vomiting. Her hematocrit was 33 per cent and her white blood cells 2,200 per cu mm. The bone marrow was largely replaced with "stem cells." Remission of symptoms followed treatment with blood transfusions, 6-mercaptopurine and steroids. Since the spring of 1958 the patient's leukemic process has remained under satisfactory control with intermittent use of steroids and 6-mercaptopurine. She entered first grade at the St. John Brebeuf School in September 1959 and has continued in school to the present.

Comment: This child's health had been good prior to the onset of leukemia except for frequent colds and sore throats. Her birth was normal. She had never been exposed to x-rays. There was no family history of cancer.

Her family moved to Niles from Chicago in the spring of 1956, and they attended the St. John Brebeuf Church regularly. This patient is the fourth youngest of five girls. At the onset of her illness in October 1957, two of her sisters were also ill, both with sore throat and otitis media. Both recovered, although a mastoid infection, which required hospitalization, developed in one. At that time this latter child was attending second grade at the St. John Brebeuf School.

Case 3. The third case of leukemia occurred in a 10-year-old white girl (K.S.) who attended fourth grade at a public school in Niles and whose family was Lutheran. Her clinical illness differed from the other seven cases, and her date of onset was less clearly defined.

In April 1958, this child was sick with the "mumps" and had a high fever for about two weeks. She lost some weight and complained thereafter of occasional "sinus" pain. In May 1958, roentgenograms of the maxillary sinuses were within normal limits. In September 1958, however, she was hospitalized because of continued pain. Minimal swelling below the right eye was noted, and roentgenograms showed destruction of the floor and lateral wall of the right orbit with opacification of the right maxillary sinus. Biopsy of the sinus revealed lymphosarcoma. A predominance of "blast" cells was found in the bone marrow, and the diagnosis was considered to be "acute lymphocytic leukemia." Her hematocrit was 34 per cent and white blood cells 5,400 per cu mm with 73 per cent lymphocytes. The child did well on treatment with steroids and 6-mercaptopurine until December 1960 when anorexia, pallor and fatigue appeared. She became acutely ill with fever, vomiting and sore throat and died on January 12, 1961, with pancytopenia and marked splenomegaly. At autopsy the lymphosarcoma mass was localized to the right maxillary sinus. As an incidental finding, calcification of the thymus was noted.

Comment: This child's birth was normal, following an uneventful pregnancy. Before the spring of 1958 she had received numerous diagnostic x-rays and had had several episodes of ill health. At 9 months of age (1949) she had "laryngitis" for which she was given "three x-ray treatments to the tonsils." At age 4 (1953) she had a transient period of fever, fatigue and leg pain. A year later she had a similar brief illness associated with cervical adenopathy. No diagnosis was made at either time, and no specific treatment was given. The patient's maternal grandfather died of gastrointestinal cancer, and a paternal aunt had chronic lymphatic leukemia.

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Her family had moved to Niles from a west Chicago suburb in 1955. She had one older sister. Her family lived within one block of the St. John Brebeuf Church and School but had no direct contact with activities there. However, the patient's closest friend was a girl of her own age who attended fourth grade at the St. John Brebeuf School in 1957-1958. These two girls frequently played and slept together on weekends. This particular companion was also the close friend of another girl in whom leukemia developed later (Case 5).

Case 4. In mid-November 1959, this 9-year-old white girl (L.B.) became abruptly ill with fever, abdominal pain and urinary frequency. Her two brothers were sick at the same time with fever, vomiting and diarrhea but recovered within a few days. The girl, however, continued to run an intermittent fever and suffered from pain in her hip. In December 1959 she was hospitalized, and after bone marrow aspiration, a diagnosis of "stem cell leukemia" was made. Her hematocrit was 37 per cent and white blood cells 7,800 per cu mm. Remission followed treatment with steroids and 6-mercaptopurine. Except for an episode of pneumonia in March 1960, she remained clinically well until the fall of 1960. She attended the St. John Brebeuf School until November 1960, but experienced increasing difficulty with anorexia and leg pain. In February 1961 she was hospitalized because of pneumonia, thrombocytopenia and hematemesis. She died on February 23, 1961. Autopsy showed widespread leukemic infiltration.

Comment: Before her illness this child had been in excellent health. Her previous medical history had been uneventful except for a tonsillectomy performed in 1957. Her birth was normal. She had never been exposed to x-rays. The patient's paternal grandfather died of cancer of the stomach.

Her family had moved to Niles from northern Chicago in November 1958, and they attended the St. John Brebeuf Church regularly. She was the oldest of three children (one girl and two boys). At the time of onset the patient was in the fourth grade and her brothers in the first and second grades at the St. John Brebeuf School.

Case 5. This 12-year-old white girl (K.E.) was in excellent health until December 1959 when she became ill with a sore throat and fever. During the following weeks she had recurrent fever and "colds." In late February 1960, a non-tender lump appeared on the left side of her neck. This lump persisted, and in April 1960 she was hospitalized. A chest roentgenogram revealed a mediastinal mass which disappeared after x-ray therapy. In June 1960, fever recurred, accompanied by "sinus" pain and splenomegaly. The child was hospitalized again, and on bone marrow examination, a diagnosis of "lymphoblastic leukemia" was made. Her hematocrit was 45 per cent and white blood cells 27,500 per cu mm with 28 per cent "blast" forms. She was treated with steroids and 6-mercaptopurine but four days later required rehospitalization because of diarrhea, vomiting, hematuria and uremia (blood urea nitrogen 195 mg per cent). Complete remission followed continued therapy. She remained asymptomatic until October 1960, when vomiting, leg pain and "sinusitis" developed. She was hospitalized with hematemesis, ecchymoses, cervical adenopathy and hepatosplenomegaly. A chest roentgenogram was within normal limits and white blood cell count was 71,000 per cu mm. She died on October 28, 1960, with severe anemia and uremia. Autopsy was not performed.

Comment: Prior to the onset of leukemia this child's health had been excellent. Her birth was normal. She had never been exposed to x-rays. There was no family history of cancer.

The patient's family had moved to Niles in 1949 from northern Chicago, and they attended the St. John Brebeuf Church regularly. She was the eldest of three children (one girl and two

boys). At the time of onset she attended the sixth grade and her two younger brothers the first and fifth grades at the St. John Brebeuf School. The patient had as a close friend the same girl who was the companion of the child in whom lymphosarcoma and leukemia developed earlier (Case 3).

Case 6. This 3-year-old white girl (M.R.) began having intermittent fever in the middle of June 1960, with temperatures ranging to 105°F. She was hospitalized in late July 1960 but her diagnosis was obscure. At that time her white blood cell count was 2,900 per cu mm with 70 per cent lymphocytes. Her hematocrit was 30 per cent, and a platelet count was 56,000 per cu mm. Bone marrow aspiration was interpreted as showing "toxic lymphocytosis." A culture of her urine grew Aerobacter aerogenes, and she was treated with numerous antibiotics without definite effect. Towards the end of August 1960 her fever subsided, and she remained asymptomatic until October 1960. At that time fever recurred, and the child was rehospitalized. Her hematocrit was 29 per cent and white blood cells 15,700 per cu mm with 72 per cent lymphocytes. Bone marrow was diagnostic of "stem cell leukemia." She responded well to therapy with steroids and 6-mercaptopurine. During the spring of 1962, however, evidence of spinal cord involvement began to appear, accompanied by the development of hepatosplenomegaly and generalized osteoporosis.

Comment: This girl enjoyed good health prior to her present illness. Her birth was normal. She had never been exposed to x-rays. Her maternal grandmother died of "leukemia" four years before her birth.

The patient's family had moved to Niles from northern Chicago in November 1957, and they attended the St. John Brebeuf Church regularly. The patient is the youngest of three children (two girls and one boy). Her 9-year-old brother was mildly ill with "three day measles" a few weeks before the onset of her illness. During the 1959-1960 school year this boy attended third grade and an older sister fifth grade at the St. John Brebeuf School.

Case 7. In early August 1960 this 13-1/2-year-old white girl (E.S.) experienced increasing fatigue followed by a spiking fever and pain in her shoulder. On hospitalization marked pallor was noted. Her hematocrit was 21 per cent and white blood cells totaled 2,500 per cu mm with 86 per cent lymphocytes. Bone marrow aspiration led to the diagnosis of "acute lymphocytic leukemia." On treatment with blood transfusions, steroids and 6-mercaptopurine remission occurred and she was able to attend the St. John Brebeuf School until Christmas 1960. At that time she and her siblings became sick with a "virus." Although the other children recovered quickly, the patient had persistent fever, diarrhea and leg pain. She was hospitalized with hepatosplenomegaly and gastrointestinal bleeding; she died on January 8, 1961. At autopsy there was widespread leukemic infiltration and staphylococcal sepsis.

Comment: Prior to the onset of leukemia this girl had enjoyed good health. Her birth was normal, and she had never been exposed to x-rays. At the age of 7 she had had a tonsillectomy. Four months before the onset of leukemia her menarche had appeared. There was no family history of cancer.

The patient's family had moved to Niles from the north side of Chicago in 1955, and they attended the St. John Brebeuf Church regularly. She was the eldest of four children (three girls and one boy). During the year before her initial illness the patient had attended the seventh grade and younger sister the fourth grade at the St. John Brebeuf School.

Case 8. This 6-1/2-year-old white girl (T.S.) became ill during the first week of August

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1960 with fever and fatigue followed by the abrupt appearance of bilateral cervical adenopathy. On hospitalization, splenomegaly and anemia were found and, on bone marrow examination, a diagnosis of "stem cell leukemia" was made. The patient was treated with blood transfusions, steroids and 6-mercaptopurine, and remission occurred. She was able to enter the first grade at the St. John Brebeuf School in September 1960 and to attend school until January 1961. At that time she contracted mumps and German measles in succession. Marked hepatosplenomegaly and cervical adenopathy appeared with a rise in white blood cell count to 350,000 per cu mm. She was hospitalized in late February 1961 with anemia, thrombocytopenia, widespread ecchymoses and gastrointestinal bleeding. She died on March 14, 1961. Autopsy examination confirmed the diagnosis of leukemia.

Comment: This child's previous health had been excellent. Her birth was normal. She had never been exposed to x-rays. Her paternal grandfather died of gastrointestinal cancer.

This patient's family had moved to Niles from Chicago in 1953, and they attended the St. John Brebeuf Church regularly. She was the second oldest of four children (three girls and one boy). In 1959-1960, the school year preceding the onset of her illness, the patient's older brother attended the second grade at the St. John Brebeuf School.

CLINICAL FEATURES

Clinical manifestations varied considerably among the eight cases (Table 3). Fever was a prominent presenting symptom. The initial white blood cell count in most cases was low or within normal limits. Three children (Cases 1, 5, and 8) exhibited cervical adenopathy, and one girl (Case 5) had transient mediastinal adenopathy. Splenomegaly was noted in five patients and hepatomegaly in four. The one non-Catholic child (Case 3) presented with lymphosarcoma of the maxillary sinus, leukemia being diagnosed later in her course. Six patients have died, the interval between onset and death ranging from six weeks to thirty-three months (Fig. 3). Two children (Cases 2 and 6) are presently living, and one (Case 2) has survived for over four years.

Most of the eight children with leukemia had enjoyed excellent health prior to onset. All were born from normal pregnancies without obstetrical or neonatal complications, and the mother of the first patient was the only mother to recall any illness during pregnancy. No moth-
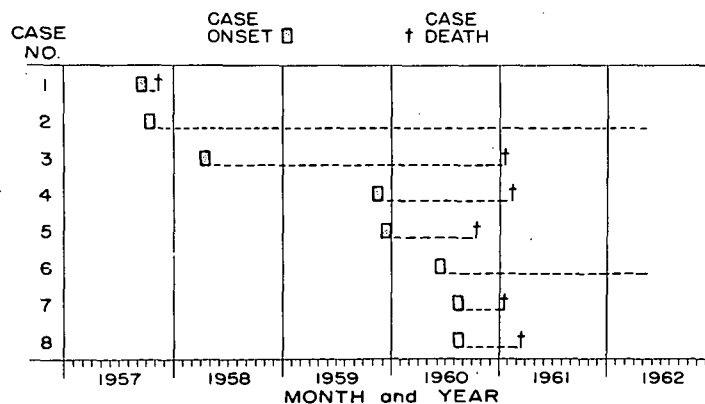

Figure 3. Leukemia among children of St. John Brebeuf Parish in Niles, Illinois, 1957-1962, by month of onset and death.

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er had received pelvimetric x-rays. The eight children were born in five different hospitals in the Chicago area. Within their families they ranged from firstborn to fourth, and at their births their mothers were from 23 to 36 years old. Four families had a history of cancer and two of leukemia, all in elderly relatives.

Each family used a different pediatrician or family physician for regular medical care. Each child had received at least three polio vaccine inoculations before the onset of illness, and the interval between the most recent Salk dose and leukemia onset varied from two to eighteen months. All the children had been fully immunized against diphtheria, pertussis, tetanus and smallpox. Results of the community survey, however, indicated that children throughout the community had similar records of complete immunization.[1]

Except for the one non-Catholic child (Case 3), none of the eight children had been exposed to any medical x-ray procedures before their illness. None gave any history which suggested unusual exposure to potentially toxic materials. All but the initial patient had had one or more of the usual childhood infectious diseases (measles, chicken pox, mumps) at varying intervals before the onset of leukemia. Only one child (Case 2) had a past history of frequent infections of the upper respiratory tract, although two of the older girls (Cases 4 and 7) had undergone tonsillectomy. Three of the eight children (Cases 3, 6 and 7) had past histories suggestive of allergy (hay fever, asthma or hives). In three families (Cases 2, 4 and 6) transient illnesses of various sorts occurred in siblings at approximately the time of onset, and in one case (Case 2) this association was striking.

EPIDEMIOLOGIC FEATURES

When arranged by month of onset the eight cases of leukemia among children fall into two general time periods (Fig. 3). The first three cases occurred during an eight month period between the fall of 1957 and the spring of 1958, and the other five during a ten month period between the fall of 1959 and the summer of 1960. No cases had their onset during the intervening eighteen months. The first period corresponded roughly with the school year 1957-1958 and the second with the school year 1959-1960.

The eight children ranged from 3 to 13-1/2 years of age at onset, and they averaged eight years old. Except for the initial patient, all were girls (Table 3). This distribution by age and sex appears to differ from general experience, in which leukemia occurs slightly more often among boys than among girls, having its greatest incidence at about 4 years of age for both sexes.[2] The small number of cases in Niles, however, limits the significance of their age and sex distribution. In addition, the apparent preponderance of cases among girls may partly reflect the fact that within the immediate families of the eight cases there were seventeen girls and only nine boys under the age of 15 at the time of onset.

A conspicuous feature of the eight cases was the absence of direct personal contact among the children or their families. Each case occurred in a different family, and none of these families were related, socially acquainted or even neighbors. When interviewed in April 1961, all eight families knew that there were other children with leukemia in the community, but almost none knew any of these other children by name.

At the time of onset four of the children with leukemia attended elementary school, one at a public school and three at the St. John Brebeuf School (Table 4). The remaining four children were of preschool age, but each had one or more older siblings attending the St. John Brebeuf

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School. Those children who were pupils at the St. John Brebeuf School, both patients and siblings of patients, were enrolled in several different grades. Some of these children were acquainted at school, but the degree of contact among them was no greater than might be expected among any children under such circumstances. The one non-Catholic child with leukemia (Case 3) was the only patient with any history of unusual interpersonal contact. This child's best friend was a girl who attended the St. John Brebeuf School and who was also the close friend of another girl in whom leukemia developed later (Case 5).

History of direct contact with the St. John Brebeuf Church and School prior to onset of disease differed widely among the eight cases. The one non-Catholic child had no such contact. The four preschool Roman Catholic children had occasional direct contact limited largely to family church attendance. The three children who attended the St. John Brebeuf School itself, of course, had considerable contact. No unusual factors of religious practice could be found peculiar to the seven Roman Catholic families.

No special characteristics distinguished the eight leukemic children or their families from other children or families in the parish area. They lived in modern, single-family homes scattered widely throughout the parish (Fig. 2a). At the time of onset of disease they had been living in Niles between one and ten years (Table 4), and all had moved to Niles from nothern sections of Chicago. Only four of the eight children had ever traveled anywhere outside the Chicago area. Their fathers' occupations varied widely and their family origins reflected the general composition of the entire community (Table 4).

In summary, examination of the eight cases of leukemia among children in Niles did not reveal any simple explanation for their occurrence. There was, however, a significant association of cases with families using one particular elementary school, and this association, together with the time pattern of case occurrence, is suggestive of a relationship to infection.

"RHEUMATIC-LIKE" ILLNESS AMONG CHILDREN

While information was being gathered concerning the eight cases of leukemia in children, the principal of the St. John Brebeuf School remarked that an "unusual" number of cases of "rheumatic fever" had occurred during recent years among the school's students. An effort was made to document this observation. In April 1961, teachers at the St. John Brebeuf School listed fourteen students alleged to have had "rheumatic fever" at some time in the past. Information from the parents of these children showed that in six illness had occurred either before 1955 or before the families concerned had moved to Niles. In the remaining eight children (three girls and five boys) illness had developed between the fall of 1957 and the summer of 1960 (Table 5). All but one were attending the St. John Brebeuf School at the time of initial symptoms.

Although there were certain features which suggested the diagnosis of acute rheumatic fever in each of these eight cases, in none could this diagnosis be established unequivocally. For this reason they are designated here as "rheumatic-like" illness. Each patient presented with fever and with pain in one or more joints. Three had objective signs of arthritis. Five had a definite history of preceding sore throat, and six exhibited an elevated ASLO titer. The severity of illness in these cases varied. Six children, however, required one or more hospitalizations, and all eight were confined to their homes for at least one month during their illness. Long-term penicillin or sulfa prophylaxis was used in all cases.

Each child recovered fully, with the exception of the initial patient. This 5-1/2-year-old

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Table 5

CLINICAL CHARACTERISTICS OF EIGHT CASES OF "RHEUMATIC-LIKE" ILLNESS AMONG CHILDREN
OF ST. JOHN BREBEUF PARISH IN NILES, ILLINOIS, 1957 - 1960

Initials	Sex and age (yr) at onset	Month and year of onset	Clinical history					
			Fever	Joint pain	Joint swelling	Sore throat	ASLO titer	Other
R.Q.	M, 5-1/2	11/57	+	+	-	+	625	Scarlet fever; Hodgkin's disease diagnosed 5/61
M.K.	M, 10	2/58	+	+	-	-	-	"Migratory rheumatism"; hospitalized three times
P.R.	F, 9	9/59	+	+	+	+	-	Sister of J.R. (Case 1)
M.S.	F, 9-1/2	3/60	+	+	-	+	2,500	Obesity; ? rheumatoid arthritis
L.C.	M, 10	4/60	+	+	-	+	625	-
G.S.	F, 11-1/2	5/60	+	+	-	+	2,500	Frequent sore throats
C.O.	M, 10	5/60	+	+	+	-	625	White blood cells 18,100 per cu. mm.
J.B.	M, 13	6/60	+	+	+	-	2,500	-

boy (R.Q.) had scarlet fever in November 1957, and thereafter experienced recurrent episodes of low grade fever, epistaxis and joint pains. In February 1959, and again in April 1960, he was ill with pharyngitis and persistent fever. In late May 1961, prominent supraclavicular adenopathy developed without other symptoms. Biopsy showed the presence of Reed-Sternberg cells and was diagnostic of Hodgkin's disease. Local x-ray therapy was given, and subsequently the patient has been asymptomatic.

This child was included in the review of "rheumatic-like" illness two months before he was found to have Hodgkin's disease. Although he did not attend the St. John Brebeuf School at the time of his initial illness, he has been enrolled there since the fall of 1958. His family moved to Niles in 1955 and have attended the St. John Brebeuf Church regularly. His closest playmate was a boy whose family lived directly across the street and whose father (A.B., Table 7) died of acute leukemia in 1957. In the same block area (Fig. 2b) lived a boy with Ewing's sarcoma (J.S., Table 6) and a woman with leukemia (R.M., Table 7). There was no history of contact with any of these patients or their families.

Table 6

CASES OF CANCER (OTHER THAN LEUKEMIA) AMONG CHILDREN OF ST. JOHN BREBEUF PARISH (ST. JB) IN NILES, ILLINOIS, 1957 - 1961

Initials	Sex and age (yr) at onset	Month and year of onset	Date of death	Church attendance	School attendance	Diagnosis
R.Q.	M, 9	5/61	-	St. JB	St. JB	Hodgkin's disease ("rheumatic-like" illness, 11/57)
J.S.	M, 11	9/57	8/3/58	St. JB	Public	Ewing's sarcoma
M.B.	F, 2-1/2	4/60	12/29/60	St. JB	2 sibs at St. JB	Sarcoma botryoides of vagina
L.M.	F, 5-1/2	7/60	-	St. JB	2 sibs at St. JB	Adenocarcinoma of thyroid

The other seven children with "rheumatic-like" illness ranged from 9 to 13 years old at the time of illness. Four were in the fourth grade at the St. John Brebeuf School and one each in grades five through seven. The third patient (P.R.) was the older sister of the young boy who died of leukemia in the fall of 1957 (J.R., Case 1). With the exception of this girl, however, none of the patients or their families had any particular personal contact either among themselves or with the families of the children with leukemia.

By month of onset the cases of "rheumatic-like" illness closely parallel the cases of leukemia, falling within the same two time periods (Fig. 4). Including the child in whom Hodgkin's disease developed later, two cases appeared during the school year 1957-1958, none during 1958-1959 and six during 1959-1960.

A similar listing of children with a history of "rheumatic fever" was compiled by school nurses in the several public elementary schools serving the parish area in order to furnish a comparison for the St. John Brebeuf School experience. Thirteen children were listed from the

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Table 7

CASES OF LEUKEMIA AMONG ADULTS OF ST. JOHN BREBEUF PARISH
(ST. JB) IN NILES, ILLINOIS, 1957 - 1961

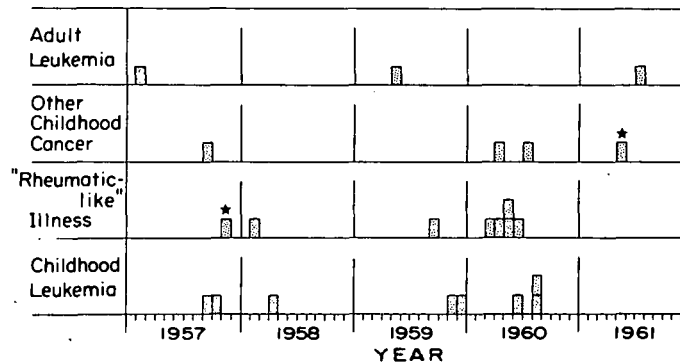| Initials	Sex and age (yr) at onset	Month and year of onset	Date of death	Church attendance	School attendance	Diagnosis
A.B.	M, 40	2/57	8/13/57	St. JB	2 sons at St. JB	Acute leukemia
R.M.	F, 59	5/59	5/28/59	other Catholic parish	-	Acute leukemia
J.S.	F, 35	7/61	5/29/62	St. JB	Sons at St. JB	Acute lcukemia with pregnancy

Figure 4. Leukemia, cancer and "rheumatic-like" illness among children, and leukemia among adults of St. John Brebeuf Parish in Niles, Illinois, 1957-1961, by month of onset. The star shows child with onset of "rheumatic-like" illness in November 1957 and appearance of Hodgkin's disease in May 1961.

the 1960-1961 public school enrollment of 2,723, compared with fourteen from the St. John Brebeuf School enrollment of 1,430. Six of these thirteen children lived within the parish area, and the illness in four of the six occurred after 1955, compared with eight for the St. John Brebeuf School. Further comparison was not feasible. Although twice as many cases of "rheumatic fever" were reported from the parochial enrollment as from a somewhat larger public enrollment, the significance of this difference is doubtful.

Among children associated with the St. John Brebeuf School, however, the coincidence in dates of onset for cases of leukemia and for cases of "rheumatic-like" illness is striking, particularly for the spring and summer of 1960. This coincidence supports the idea that the occurrence of leukemia among children in this community may have been related to the occurrence of some infectious process.

OTHER CANCERS AMONG CHILDREN

Three fatal cases of cancer other than leukemia occurred among children under the age of 15 in Niles during the years 1956-1960. Two of these children died of Ewing's sarcoma and one of sarcoma botryoides of the vagina. One of the children with Ewing's sarcoma was an 11-year-old girl who died in Niles in June 1958, but who did not move to Niles until after the onset of her illness in September 1957.

The remaining two children (Table 6) lived within the St. John Brebeuf Parish area at the time of onset of their disease. Both were from Roman Catholic families. One was an 11-year-old boy (J.S.) in whom Ewing's sarcoma developed in early September 1957, and who died in August 1958. This child was the eldest of seven children; he attended a public school in Niles. His family attended the St. John Brebeuf Church regularly. They had moved to Niles in 1954 and lived in the same block area as the child with Hodgkin's disease and two adults with leukemia (Fig. 2b).

The second child with cancer was a 2-1/2-year-old girl (M.B.) who died in December 1960 of sarcoma botryoides of the vagina; she first became ill in April 1960. Her family had moved to Niles before her birth. They attended the St. John Brebeuf Church regularly, and at the time of onset two older siblings attended the St. John Brebeuf School.

These cases probably represent all fatal cases of cancer other than leukemia to occur among children in Niles between 1956 and 1960. A survey of similar completeness was not possible for non-fatal cancer among children, although inquiry about such illness was made in both the public and the parochial schools. The only case discovered, in addition to the boy with Hodgkin's disease already described, was a case of thyroid cancer (Table 6) in a girl whose family attended the St. John Brebeuf Church and whose two older brothers attended the St. John Brebeuf School. In July 1960, this 5-1/2-year-old girl (L.M.) was found to have papillary adenocarcinoma of the thyroid. Following surgical resection of this tumor she has been well without evidence of recurrence.

In none of these cases, with the exception of the child with Hodgkin's disease, was there any history of direct personal contact with other patients with cancer or leukemia. The families of each attended the St. John Brebeuf Church, and children from two attended the St. John Brebeuf School at the time of onset of disease. The case of Ewing's sarcoma had its onset in the fall of 1957 while the cases of sarcoma botryoides and thyroid cancer both occurred in the spring and summer of 1960 (Fig. 4). These dates of onset coincide with the onset pattern already observed for cases of leukemia and "rheumatic-like" illness in children.

LEUKEMIA AMONG ADULTS

Three deaths from leukemia among adults were recorded in Niles between 1956 and 1960, and an additional case was diagnosed in the summer of 1961. One of the three fatal cases occurred in a 60-year-old man who died in 1960 of chronic lymphatic leukemia first diagnosed in 1955. He lived alone in the southern section of Niles and had no known contact with the St. John Brebeuf Parish.

The other three adults with leukemia all lived within the St. John Brebeuf Parish area. All were Roman Catholic, and two had children attending the St. John Brebeuf School at the time of their illness (Table 7).

The first case occurred in a 40-year-old man (A.B.) who became ill in February 1957 and

who died seven months later. This patient was considered to have either "acute myeloid" or "acute monocytic" leukemia. He had moved to Niles in 1955 with his wife and three sons. They attended the St. John Brebeuf Church regularly, and his two older sons were pupils at the St. John Brebeuf School. This man lived in the same block as three other persons with cancer, one of whom was the child in whom Hodgkin's disease developed later (Fig. 2b). This child and the youngest son of the patient (A.B.) frequently played together.

The second adult with leukemia was a 59-year-old woman (R.M.) who became ill abruptly in May 1959 and died within a month. The diagnosis was made of either "acute monocytic" or "acute lymphatic" leukemia. The patient had moved to Niles with her family five months before her death, and she lived in the same block as the man with leukemia and the two boys with other cancers (Fig. 2b). She and her family were Roman Catholic, but they attended a parish other than St. John Brebeuf. None of her five children had attended the St. John Brebeuf School.

The most recent case of leukemia in an adult occurred in a 35-year-old woman (J.S.) whose first symptoms appeared in July 1961; she died on May 29, 1962. "Acute monocytic" leukemia was diagnosed, and the disease was coincident with pregnancy. The details of this patient's clinical course and management are discussed by Ravenna and Stein in a separate publication.[3] The patient and her family had moved to Niles in 1957. They attended the St. John Brebeuf Church regularly, and her one older son was a pupil at the St. John Brebeuf School.

There was no history of direct contact among these three patients or their families. In addition, the only association with other Niles families in which leukemia or cancer occurred was between the child with Hodgkin's disease and the son of the man with leukemia. The dates of onset of the three adults with leukemia do not closely parallel the pattern observed for childhood leukemia in children (Fig. 4).

CHILDHOOD MORTALITY FROM OTHER CAUSES

Childhood mortality in Niles during 1956-1960 from causes other than leukemia and cancer was examined through death certificate data and compared with similar data in neighboring towns. No significant differences were found except among deaths associated with congenital malformation. In Niles such deaths showed a predominance of cardiac defects.

Congenital heart disease. Seven of eight deaths (88 per cent) associated with congenital malformation in Niles were cardiac in type compared with 19 of 61 (31 per cent) in four neighboring towns (Table 8). These seven deaths yielded a mortality rate for congenital heart disease in Niles which was significantly increased over comparable rates in neighboring towns, with the exception of Des Plaines, Illinois. In this latter town a rate equivalent to that in Niles was observed; however, this was accompanied by a corresponding increase in mortality associated with all types of congenital malformation.

The seven cases of congenital heart disease in Niles were all in Roman Catholic families, and four of these families lived within the St. John Brebeuf Parish area. The one non-cardiac case, a girl with multiple skeletal anomalies who died at birth in May 1960, was from a non-Catholic family living within the parish area. The diagnosis in each of the four cardiac cases was confirmed either at surgery or at autopsy. They included two boys, one born in July 1955 with tetralogy of Fallot and one born in December 1956 with ventricular septal defect associated with polycystic kidneys; and two girls, one born in July 1957 with hypoplasia of the aortic tract and one born in April 1960 with transposition of the great vessels. None of these four chil-

Table 8

MORTALITY ASSOCIATED WITH CONGENITAL HEART DISEASE AMONG
CHILDREN UNDER AGE 15 IN NILES, ILLINOIS,
AND VICINITY, 1956 - 1960

Area	Population under age 15 (1960 census)	Deaths associated with congenital malformation		Deaths associated with congenital heart disease		
		No.	Rate*	No.	Per cent†	Rate*
Des Plaines	11,952	28	46.9	11	39	18.4
Lincolnwood	3,654	5	27.4	1	20	5.5
Morton Grove	7,995	6	15.0	1	17	2.5
Niles	7,076	8	22.6	7	88	19.8
Skokie	20,474	22	21.5	6	27	5.9

*Average annual mortality per 100,000.

†Per cent of deaths associated with congenital malformations.

dren or their siblings were enrolled at the St. John Brebeuf School, although their families attended the St. John Brebeuf Church.

Miscellaneous cases. Three additional deaths which occurred among children in Niles during 1956-1960 warrant brief mention. In August 1960 a 5-year-old boy died of acute leukemia which was first diagnosed in November 1959 at which time he was living in Chicago. His family moved to Niles in February 1960, and attended the St. John Brebeuf Church. Although this boy was enrolled to enter the St. John Brebeuf School in the fall of 1960, he had no history of direct contact with parish activities.

An 8-year-old girl from a non-Catholic family which lived within the St. John Brebeuf Parish area died of aplastic anemia in October 1960. Her illness began in April 1960, and followed treatment of an infection with Chloromycetin.® This child had no direct contact with the St. John Brebeuf Church or School.

The third death occurred in August 1956 in a 2-1/2-year-old boy from a Roman Catholic family which lived within the St. John Brebeuf Parish. This child was mongoloid and died after a three month illness which was marked by hypochromic anemia and hepatosplenomegaly. The diagnosis of leukemia associated with mongolism was not entirely ruled out but would seem unlikely.

OTHER STUDIES

Levels of background radiation were measured in the buildings of the St. John Brebeuf Church and School and in various areas throughout the community, including the homes of several of the children with leukemia. In each instance readings were of a low normal level.

A survey of veterinarians in the vicinity of Niles revealed no unusual prevalence of leukemia among domestic animals. The only case reported from this inquiry was in a 9-year-old bassett hound from Des Plaines, Illinois, which died in June 1960 after a two month illness.

DISCUSSION

Evidence suggesting that cases of leukemia may occur in clusters within communities has been presented on several occasions in the past.[4-6] A recent study in the Buffalo, New York area[7] examined cases of childhood leukemia by place of residence and by time of occurrence. In this study, clusters, consisting of two to three cases each, were found with a frequency of "suggestive significance." In other communities in the United States apparent concentrations of cases of leukemia have also been investigated in recent years.[8] None of these case concentrations, however, has been shown conclusively to exceed the realm of chance occurrence.

The cluster of eight cases of childhood leukemia among children in Niles cannot reasonably be attributed to the effects of random distribution. These cases constitute a clearly defined micro-epidemic which occurred within a particular community and which affected a particular segment of that community's population. Although obscure in etiology, this micro-epidemic presents several distinct epidemiologic features.

The association of cases with the St. John Brebeuf School, the time pattern of their onset and the parallel appearance of "rheumatic-like" illness all suggest a relationship in this community between leukemia in children and some infectious process. If such a relationship were true, differences in community activities leading to differences in interpersonal contact might help to explain the concentration of cases of leukemia among families using the St. John Brebeuf School. The opportunities for increased interpersonal contact among these particular families would facilitate or intensify person-to-person spread of infectious agents and might lead to patterns of illness within these families different from those experienced by the rest of the parish community. The findings of the community survey suggest that such differences did exist in the occurrence of such common childhood illness as measles and chicken pox.[1]

Although the pattern of leukemia among children in Niles implies a relationship to some infectious process, there is little evidence to indicate what this process might be. If it is hypothesized that the cases of leukemia were caused by a specific infectious agent, the absence of contact among the children with leukemia and the presence of no more than one case in any one family would indicate an agent of high infectivity but low pathogenicity. In such a situation the agent's ability to produce leukemia could depend upon a particular dose threshold which might be reached more often among families using the St. John Brebeuf School than among other families on the basis of greater and more frequent exposures to infectious agents.

As an alternate possibility, the production of leukemia, with or without a specific infectious agent, might depend basically on the prevalence of a particular non-specific infection or combination of infections at a particular time. Such special disease conditions might prevail among families using the St. John Brebeuf School without similar conditions being present simultaneously in the rest of the community.

The pattern of "rheumatic-like" illness in the St. John Brebeuf Parish suggests that the cases of leukemia in Niles may represent a sequel to infection in the manner in which rheumatic fever may follow streptococcal disease. This association of leukemia with "rheumatic-like" illness recalls the observations of Abbatt and Lea[9] who demonstrated an increased incidence of leukemia in a series of patients with ankylosing spondylitis treated with x-rays and who indicated the possibility that leukemia may be associated with this rheumatic process independent of x-ray therapy. Pursuing this hypothesis, these same authors later presented evidence that a past history of some type of rheumatic disease is significantly more frequent among patients

with leukemia than among control patients.[10]

Along a similar vein, Manning and Carroll[11] have presented data showing that manifestations of allergy (hay fever, asthma, hives) may be more common among children with leukemia than among control children. Among the cases in Niles as a whole, however, no such unusual history of allergy or hypersensitivity was found.

Although cases of leukemia in adults and of cancer other than leukemia in children were few in Niles, the concentration of such cases among Roman Catholic families, particularly among families using the St. John Brebeuf School, corresponds with the pattern observed for cases of leukemia in children. A similar concentration among Roman Catholic families was also found for childhood deaths associated with congenital heart disease. In view of the known teratogenic effects of certain infections during pregnance,[12] the latter observation may constitute further evidence suggesting the operation of some unusual infectious process in Niles during the period of leukemia occurrence.

The appearance of cases of leukemia in Niles coincided with a period of rapid population expansion within the St. John Brebeuf Parish area. Since such a young, growing community tends to attract young, growing families from many different neighborhoods with different disease experience, the expansion of this community may well have been accompanied by abruptly changing patterns of disease. The occurrence of leukemia in the St. John Brebeuf Parish area may represent such a changing pattern of disease, the product in some manner of this community's unusual period of growth and adjustment.

While the concentration of eight cases of leukemia in Niles is unusual, such a cluster of cases should not be considered unique. Leukemia is a relatively rare disease, and a few related cases which occur within a community over a period of several months or years might never be detected unless specific inquiry were made. In the light of the experience in Niles further search for case clusters, coupled with detailed study of cases of leukemia and other cancers as they occur within particular communities, represents a promising approach to understanding the etiology of these diseases.

SUMMARY

Between 1957 and 1960 eight cases of leukemia occurred among children living in one residential area of Niles, Illinois. Seven of these eight children were from Roman Catholic families, and each of these seven either attended or had older siblings who attended the community's one parochial elementary school.

These cases constituted a significantly increased incidence of leukemia in children for this town, and their association with a single school exceeded chance expectation. The eight cases occurred within two separate periods of time, and were accompanied by a "rheumatic-like" illness among pupils in the same parochial school.

Within this same community, cases of leukemia in adults and of cancers other than leukemia in children were found limited to Roman Catholic families, four of whom lived within a single block area. An unusual childhood mortality associated with congenital heart disease was observed in which cases were also limited to Roman Catholic families.

Consolidation in the parochial school and rapid population expansion in the community may have been contributing factors in this unusual occurrence of leukemia. Although the exact cause of these cases was not identified, their association with one particular school, the time pattern

of their occurrence and the parallel appearance of a "rheumatic-like" illness suggest an etiologic relationship to infection.

ACKNOWLEDGMENT

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A NUCLEAR NEEDLE FOR USE IN NEUROSURGERY[*]

By

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The beta emitting radioactive isotopes provide lesions of predictable size in neural tissue.[1-3] The range of these particles is constant for each particular isotope, and the dose delivered depends upon the rate of emission, the distance from the source, and the duration of contact with the tissue. As the activity of the source may be kept constant, the size of the lesion can be accurately controlled (up to its maximum) by varying the duration of contact.

This clinical simplicity is somewhat offset by the inconvenience of obtaining from a nuclear reactor a new supply of isotope for each clinical application, for it is the rule that those isotopes with the more penetrating beta emissions have the shorter half-lives. There is, however, one useful combination, strontium-90 yttrium-90. Strontium-90 is one of the more abundant fission products of uranium-235. It is not ordinarily available in isotopically pure form because of contamination with other stable strontium fission products. The maximum available specific activity is about one-third the theoretical value of 145 curies per gram. Strontium-90 decays with a 28-year half-life by emission of a beta particle of moderate energy. (E_{max} 0.54 MEV) to yttrium-90 which decays in turn to zirconium-90 with a half-life of 2.67 days by emission of a high energy beta particle (E_{max} 2.27 MEV) which has a range of about 11 mm in water. Thus a Sr^{90} - Y^{90} source delivers a high energy beta on a virtually permanent basis. Its long half-life makes contamination by the material particularly dangerous (maximum permissible body burden of Sr^{90} is 1 μc) so that for clinical use it must be enclosed in an hermetically sealed applicator.

The Sr^{90} - Y^{90} needle (Fig. 1). Construction of such a source was undertaken by one of us (P.V.H.) at the Argonne Cancer Research Hospital in August, 1961. Strontium sulfate was chosen as the most suitable stable compound which could be easily prepared and handled. Two hundred millicuries of Sr^{90} as Sr Cl$_2$ was obtained from Oak Ridge National Laboratory. The volume of
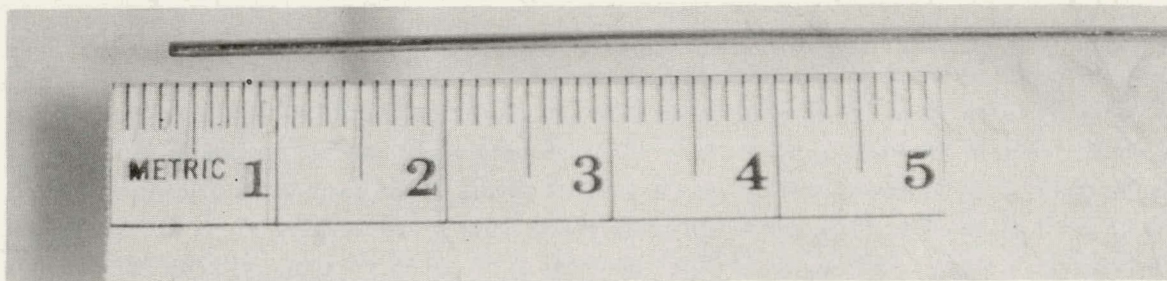

Figure 1. This small diameter No. 19 needle fits into a thin wall No. 17 introducing sheath. No. 19 needle wall has beta absorption capacity of 1.5 mm tissue, approx.

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this was reduced to 0.1 ml, and 0.1 ml of concentrated H_2SO_4 was added to produce the sulfate. Ten ml of ethanol added to this resulted in a coarse granular non-adherent precipitate. This was washed with three 10 ml portions of diethyl ether to remove the excess H_2SO_4 and transferred as a suspension in ether to a steeply angled, polished, stainless steel funnel soldered to a standard 4 inch, number 19 gauge needle, the tip of which had been welded shut, radiographed and leak tested. Centrifuging this funnel carried a substantial portion of the precipitated strontium sulfate down to the tip of the needle. The ether and remaining precipitate were removed, and the residual ether was driven off by gentle heat. The dry precipitate in the tip of the needle was tamped down firmly with a wire obturator and was sealed in place by tamping down on top of it a small plug of soft indium metal. The remaining portion of the needle was freed from significant contamination by washing with water. The needle was unsoldered from the apex of the funnel, the lumen occluded with a steel wire obturator, and a conventional hub soldered in place to provide a handle. All traces of remaining strontium were collected together and assayed, and, by difference, 54 millicuries was determined to be in the tip of the needle. It is estimated that the wall thickness of the needle (19 gauge) has an absorbing capacity equivalent to about one and a half millimeters of tissue.

Physical assay. Since the entire energy of the beta particles (E = 0.89 MEV) is dissipated in a very small volume, a very intense, very localized radiation field is produced in the surrounding absorbing material (Figs. 2 and 3). The absorption of the Y^{90} beta radiation is to a
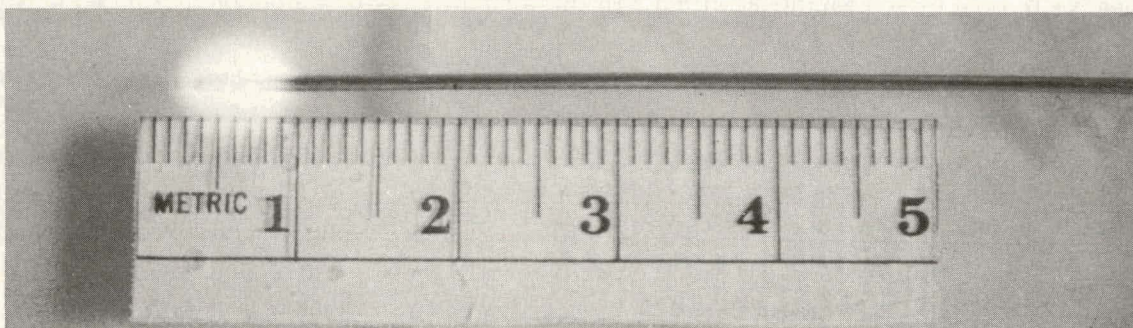

Figure 2. High energy beta particles travel faster in water than light travels in water. A glow is emitted (Cerenkov effect) in the area of most intense radiation (Sr^{90} Y^{90} 54 mc).

first approximation exponential with a half-value layer of about 150 mg per sq cm, or 1.5 mm in tissue. Combining this with the inverse square attenuation, the radiation dose intensity falls off by a factor of about 10 for each 2 millimeters from the source. These measurements were made in a lucite phantom against a U_3O_8 standard (4 and 5), and the observed gradient and dose rates are shown in Figure 4.

Biological assay. One hundred lesions were made in 25 animals (19 dogs and 6 cats) anesthetized with Nembutal. Periods of irradiation were 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, and 120 minutes. Animals were sacrificed at the 12th and 18th hour and on the 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, 8th, 10th, 12th, 20th, 30th, 60th, 90th and 240th day after irradiation. The brains were fixed by immersion in formalin. After 9 days they were sectioned and the necrotic areas were measured by macroscopic methods. They were then prepared for histological exam-
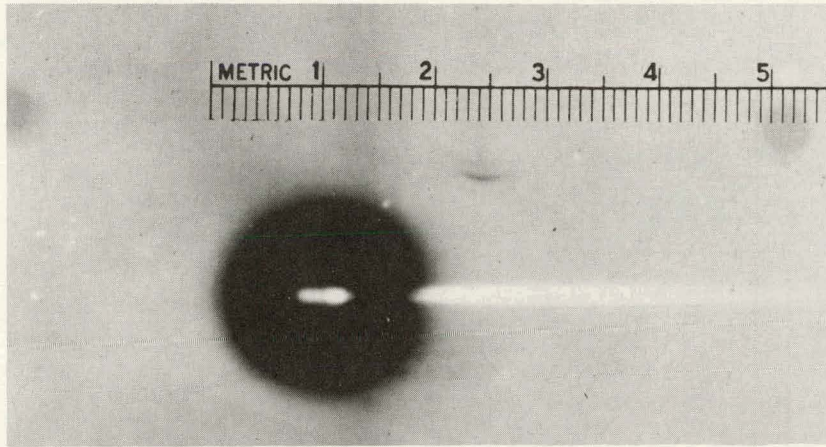
Figure 3. Autoradiogram shows maximum range of
beta particles (in wax) (Sr^{90} Y^{90} 54 mc).

ination, using the hematoxylin and eosin stains. Selected specimens were also studied by Kluver
stain.

Macroscopic examination. The gross lesions were circular in cross section and oval in
longitudinal section. The measurements given are those of the formalin-fixed material. Depth
and width increased with duration of exposure up to 60 minutes, as shown in Figures 5 and 6.
It is noted that those lesions resulting from radiation periods shorter than 30 minutes are not
hemorrhagic, but that those resulting from periods longer than 30 minutes are hemorrhagic.
This hemorrhage causes irregularity in the size of the lesion from one animal to another,
whereas non-hemorrhagic lesions (for a given dose) are identical from animal to animal.

Histological examination. Three zones may be distinguished (Figs. 7 and 8). In the center
is a zone of absolute necrosis (Figs. 9, 11 and 12) in which all cell and fiber detail is quickly
lost. This is surrounded by a middle zone in which cells and fibers are recognizable, but the
damage is so severe that one cannot imagine that physiological function could be either pre-
served or restored (Figs. 9, 10, 11, 13 and 14). The outer zone is one of edema in which physi-
ological function is probably preserved or to which it may be restored (Figs. 10 and 11). There
is a clear boundary between the central and middle zones. The boundary between the middle
and outer zones, and the peripheral boundary of the outer zone are indistinct and quite arbi-
trary. The inner necrotic zone develops rapidly and reaches its maximum between the 3rd and
5th days (Fig. 8). It is still prominent by the 90th day, but by the 240th it is replaced by a cyst
(Figs. 5 and 15). The middle degenerative zone reaches its maximum about the 12th day and is
still recognizable by the 90th. The outer zone of edema reaches its maximum by the 7th to 10th
day and has virtually disappeared between the 20th and 25th days. Figure 8 represents the width
of these zones from the center of the needle in relation to the time interval after radiation for a
15-minute radiation dose. Similar graphs may be made for other periods of radiation. The ra-
dius of the necrotic zone varies with the dose as in Figure 6, but the width of the areas of de-
generation and of edema are approximately the same for all.

During the first three days of the development of the central necrotic zone, the advancing
edge is characterized by the appearance of tiny hemorrhages (Fig. 9). By the 5th day there is
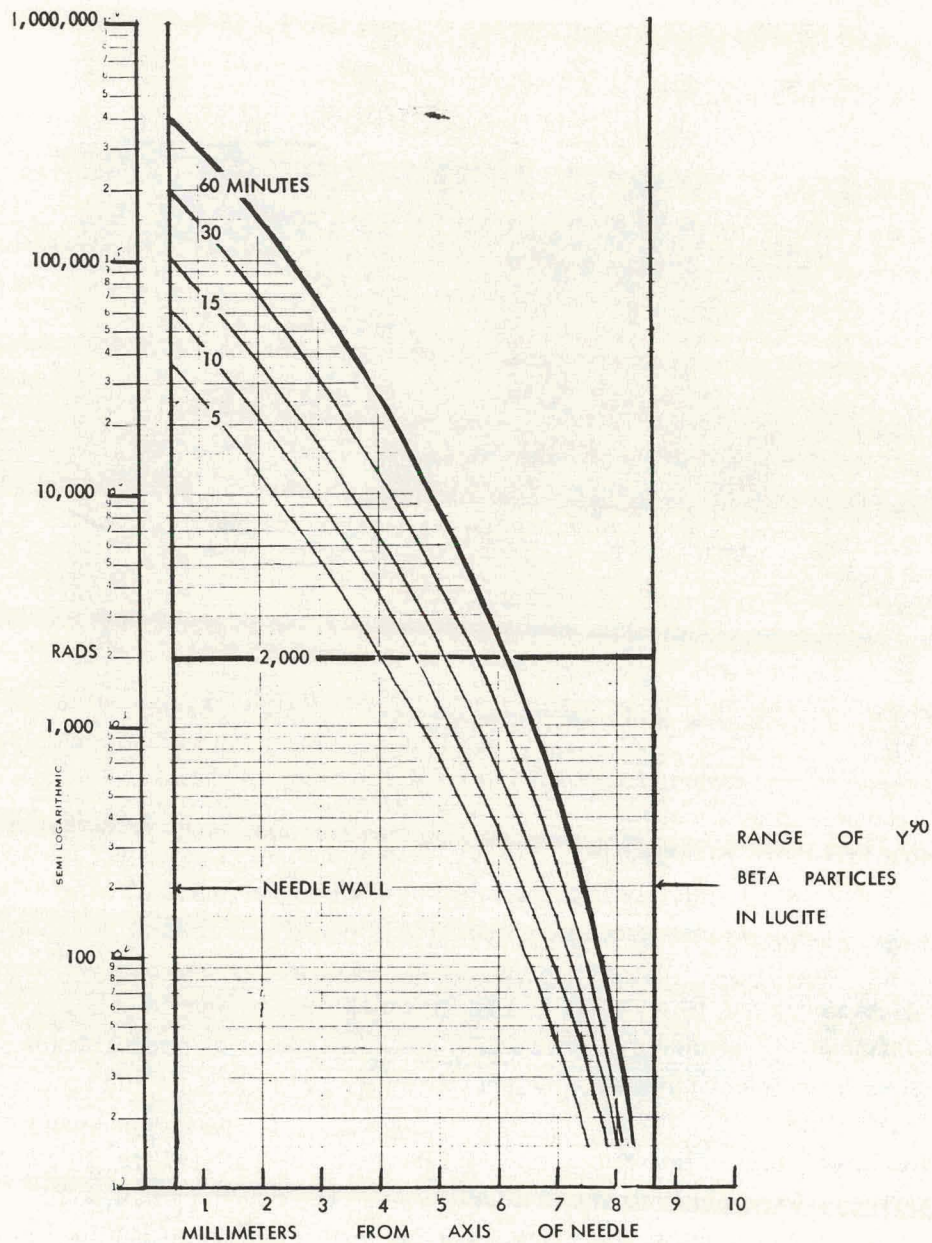
Figure 4. Semilogarithmic graph showing dose delivered at increasing distances from the axis of the needle for 5, 10, 15, 30 and 60-minute periods of exposure. The 2,000 Rad line marks off the lethal dose in neural tissue (Sr^{90} Y^{90} 54 mc).

a ring of hemorrhage at the boundary zone between the necrotic and degenerative zones (Figs. 5 and 10). In the center of the necrotic zone those hemorrhages which develop as necrosis advances are seen scattered among the degenerated cells and fibers (Figs. 11 and 12). Blood vessels in the center are thrombosed and hyalinized. Blood vessels at the periphery remain intact after nerve cells have died (Fig. 11), but in time they too become hyalinized (Fig. 13). For lesions at periods of more than 30 minutes the hemorrhage is so extensive that the whole center
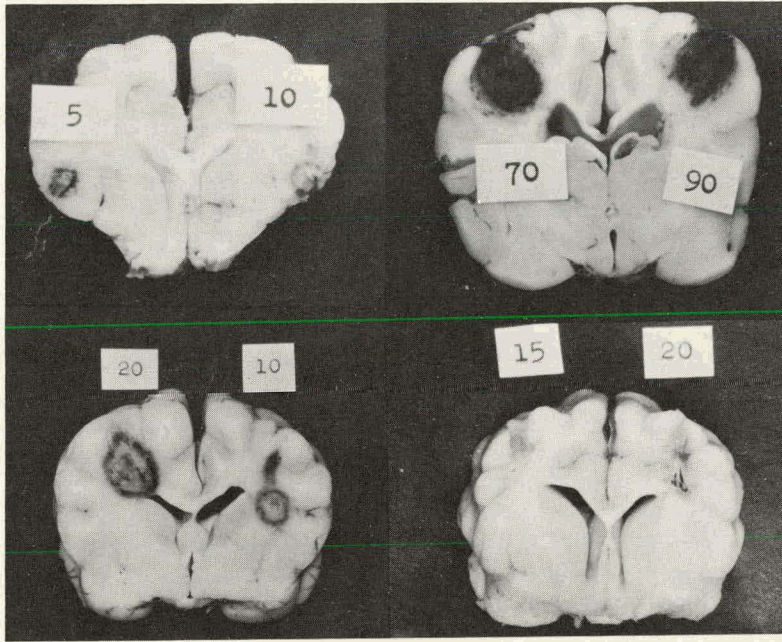
Figure 5. Sr^{90} Y^{90} 54 mc lesions in animal brains. Numbers indicate minutes of exposure. The animal represented in the bottom right was sacrificed after 240 days. All others were sacrificed on the 5th day.
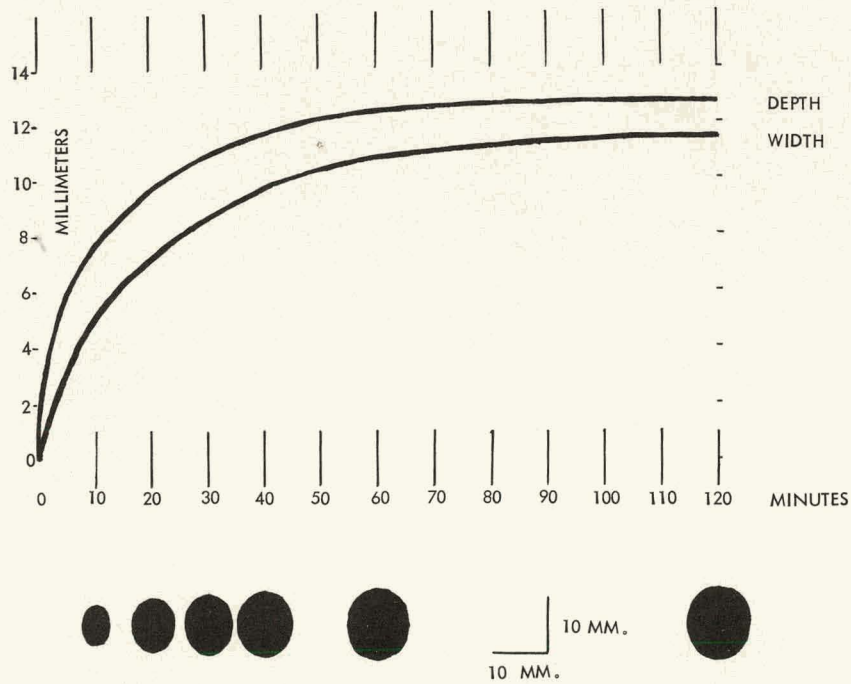

Figure 6. Graph representing increasing size of lesions with increasing duration of exposure. The measurements for lesions of less than 30 minutes' duration are absolute. The measurements for those of more than 30 minutes' duration are average. (Sr^{90} Y^{90} 54 mc).
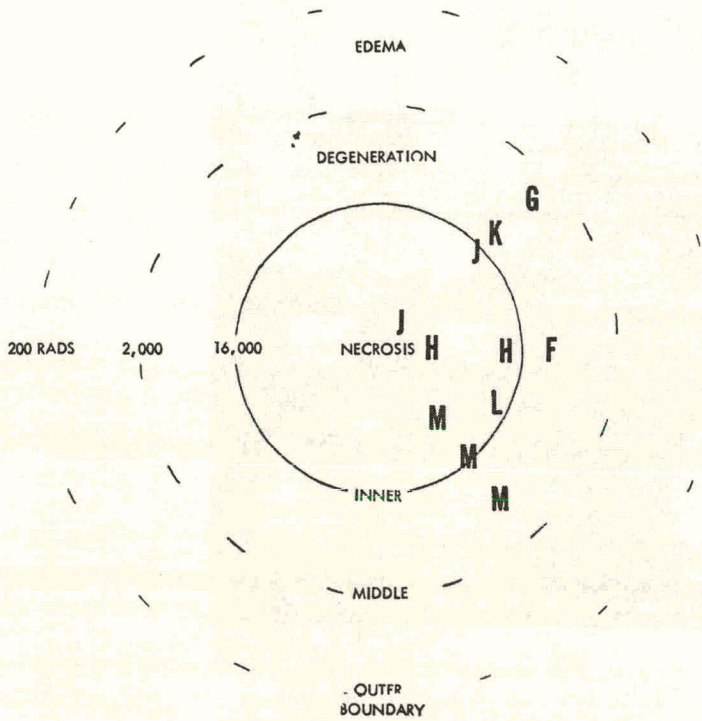
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Figure 7. Concentric zones of necrosis degeneration and edema. F, degenerate cells. G, normal blood vessels. H. hemorrhage. J. hyalinized blood vessels. K, edema around blood vessel. L, polymorph and lymphocyte infiltration. M, macrophages.
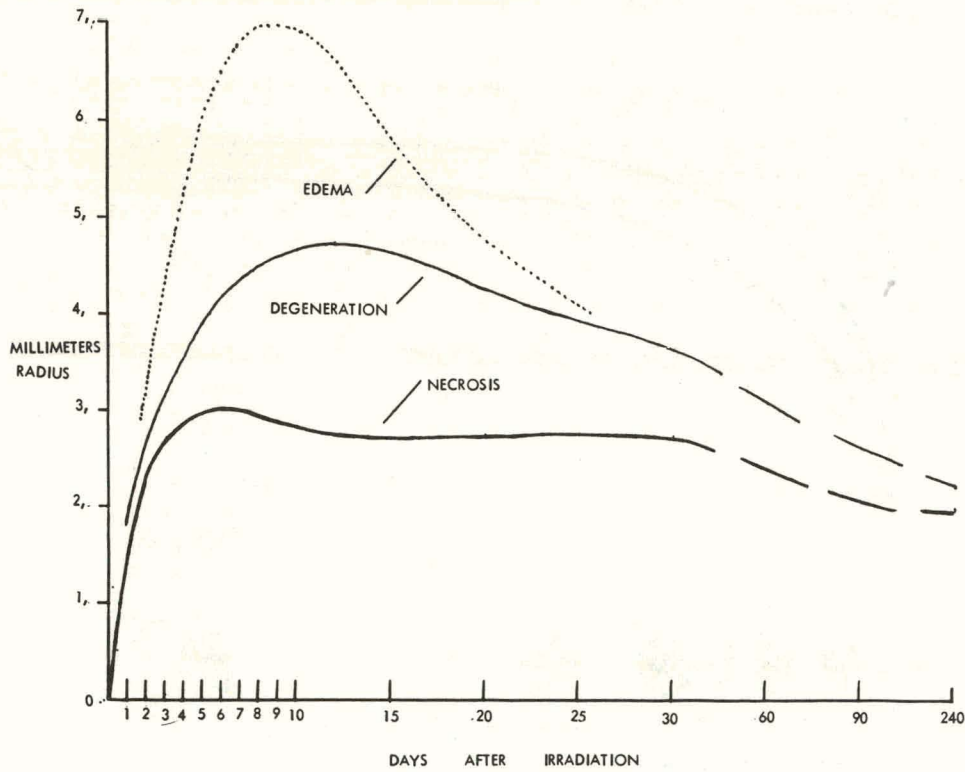

Figure 8. Graph showing evolution over a period of 240 days of the zones of necrosis, degeneration and edema that result from a 15-minute exposure ($Sr^{90} Y^{90}$ 54 mc).

Figure 9. Sr^{90} Y^{90} 54 mc, 15 min, sacrificed after 24 hours. H and E x 60. A, zone of necrosis. B, zone of degeneration. D, inner boundary. F, degenerate cells. H, hemorrhages. N, needle tract.
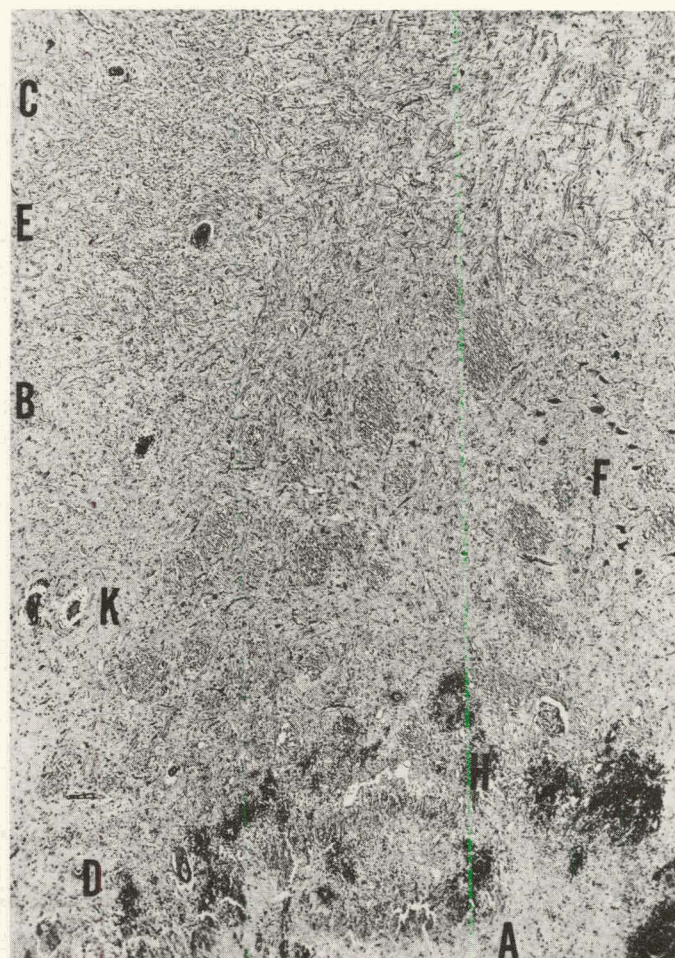

Figure 10. Sr^{90} Y^{90} 54 mc, 15 min, 5th day. H and E x 47. A, necrosis. B, degeneration. C, edema. D, inner boundary. E, middle boundary. F, degenerate cells. H, hemorrhage. K, edema, around blood vessels.
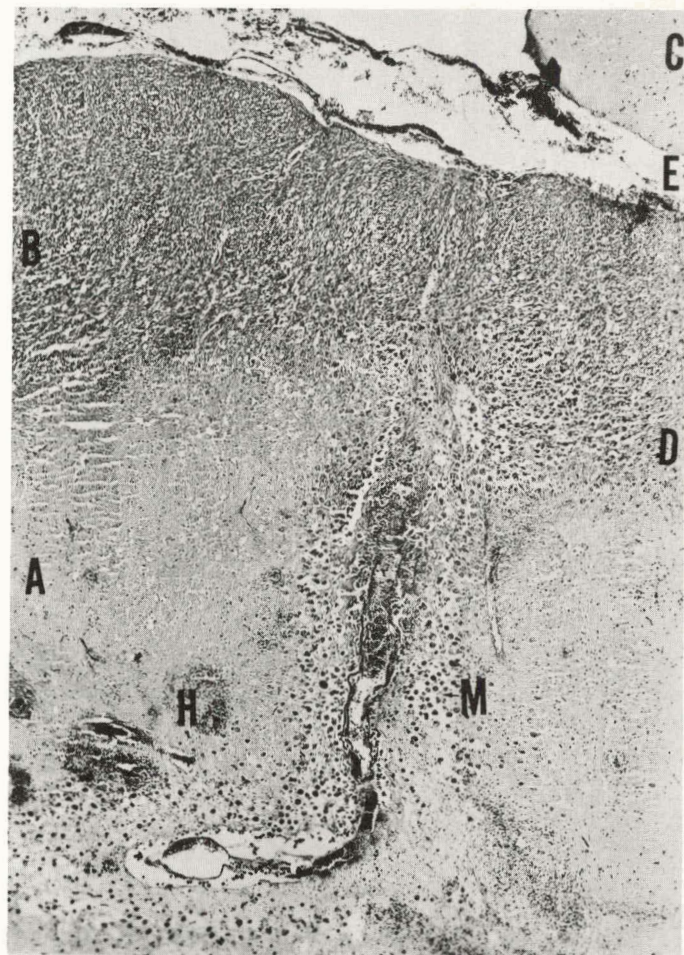
Figure 11. Sr^{90} Y^{90} 54 mc, 15 min, 12th day. H and E x 60. A, necrosis. B, degeneration. C, edema. D, inner boundary. E, middle boundary. H, hemorrhage. M, macrophages around surviving blood vessels.
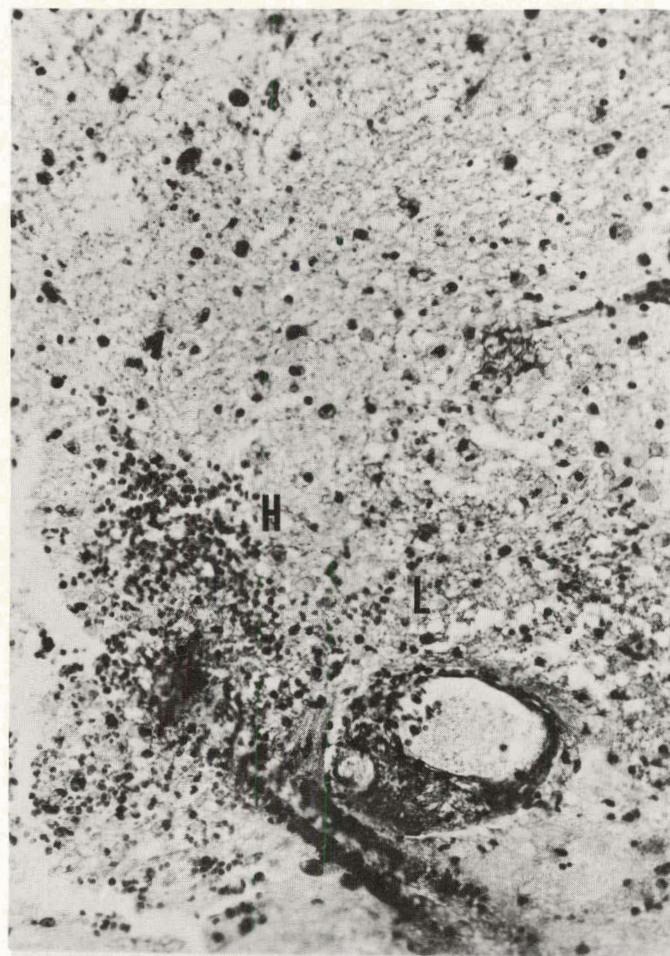

Figure 12. Sr^{90} Y^{90} 54 mc, 15 min, 3rd day. H and E x 275. Central necrosis showing hemorrhages. L, with polymorph and leucocyte infiltration.

Figure 13. Sr^{90} Y^{90} 54 mc, 15 min, 60th day. H and E x 160. A, necrosis. B, degeneration (looks much worse than similar area in Fig. 9, 3rd day). D, inner boundary. J, hyalinized blood vessel. K, blood vessel. M, macrophages.
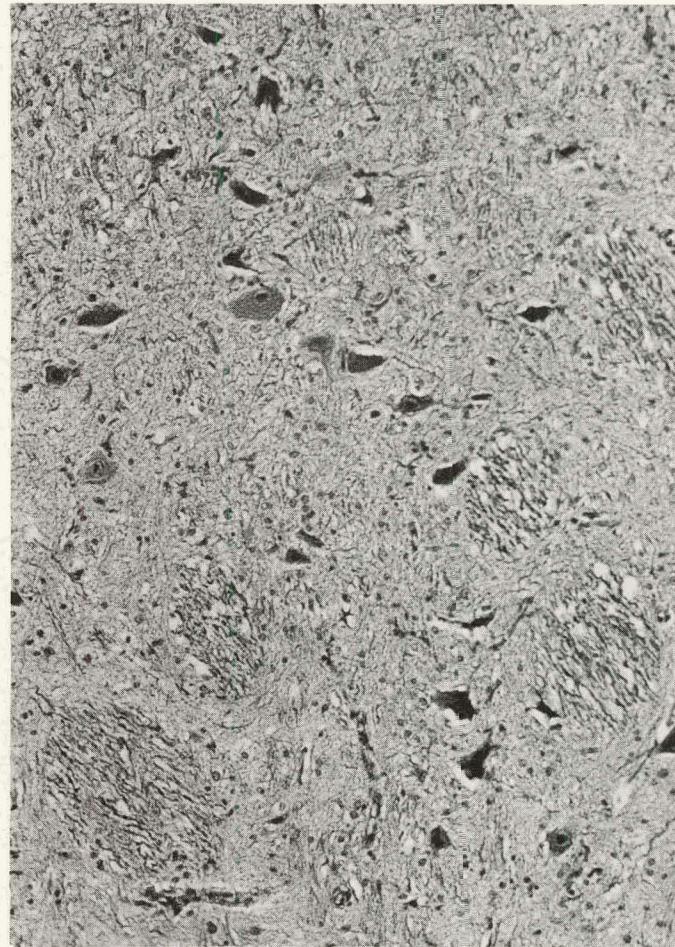

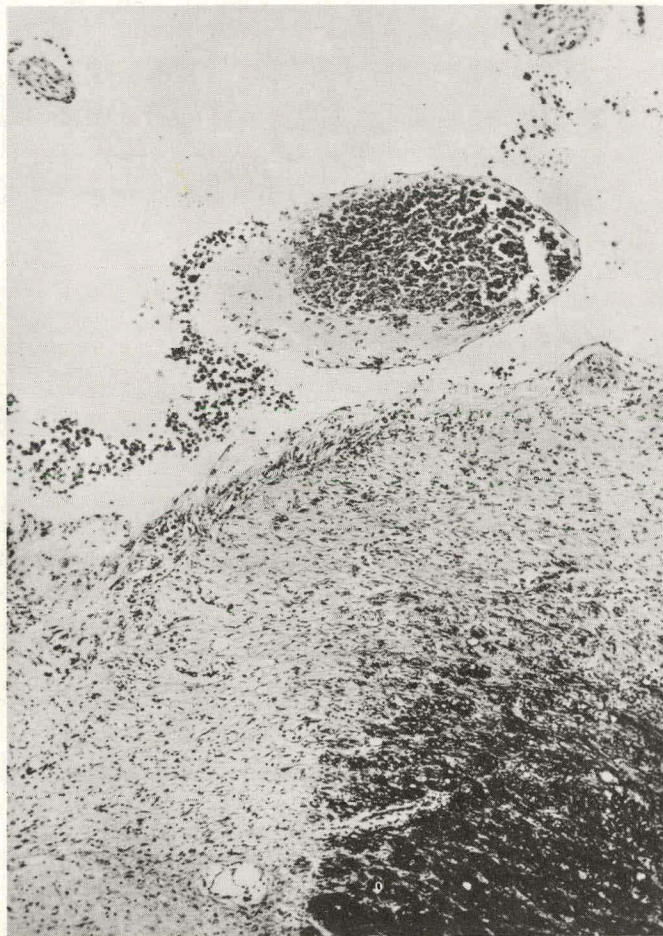Figure 14. Sr^{90} Y^{90} 54 mc, 15 min, 3rd day. H and E x 160. Shows that cells F of Figure 9 are definitely degenerating.

Figure 15. Sr^{90} Y^{90} 54 mc, 15 min, 240th day. Kluver and Barrera. Cyst still contains some macrophages. The wall of fibrillary astrocytes quickly passes into normally myelinated white matter.

seems occupied by red cells and the entire lesion may become quite irregular (Fig. 5). Blood vessels in the zone of degeneration become dilated and are surrounded by a clear area, which represents edema (Fig. 10). Hyalinization has not been noted in them during the period of observation (Fig. 13).

There is an infiltration of polymorphonuclear lymphocytes into the necrotic zone (Fig. 12), especially in those with the more marked hemorrhages. This is not a very prominent infiltration, but it is quite noticeable by the 3rd day. On the 7th and 8th days there is an extensive macrophage infiltration of the inner boundary. These cells also aggregate around those vessels that are spared in the periphery of the necrotic zone. From the boundary area and from these blood vessels they infiltrate into the necrotic and degenerative zones (Figs. 11, 13). Their activity continues to the 90th day, but by the 240th day when cyst formation is virtually complete a few are still present (Fig. 15).

The lesions of the 240th day specimens are as a rule cystic with a diameter of 6 mm or

less (Figs. 5, 15). The adjacent lateral ventricle is dilated if closely situated. The cavity has clear-cut sides and sometimes contains a brownish yellow, gummy fluid. Some hemosiderin and fat-laden cells persist. The wall is formed of a layer of fibrillary astrocytes of about 0.5 mm thickness. Its blood vessels show some thickening of their walls and are sometimes surrounded by macrophages. Beyond this wall neural tissue appears normal.

DISCUSSION

Comparison of Graphs 4 and 8 will show that for a 15-minute exposure approximately 16,000 rads are delivered at 3 mm from the axis of the needle, and that this dose produces early complete necrosis. Two thousand rads are delivered at 5 mm from the axis (for the same exposure) and this amount, when given in one dose, is usually assumed to prove lethal to nerve tissue. This 5 mm line corresponds very closely to our arbitrary outer boundary of the zone of degeneration. The arbitrary nature of this boundary, errors due to swelling of the living tissue, and to fixation after sacrifice, make it difficult to confirm the lethal range in living tissue with exactitude, but it certainly approximates very closely to the 5 mm line.

It is also difficult to state exactly when death occurs in the degenerative zone. Studies on palladium-109[2] showed a much less prominent zone of degeneration. In those lesions the macrophages were quickly able to remove the zone of necrosis, and lesions of 5 or 6 mm diameter had become completely cystic by the 60th day. In contrast, the persistence of the zone of necrosis in the strontium-yttrium lesions suggests that continued destruction within the degenerating zone adds to the necrotic zone as quickly as macrophages remove it. The maximum width of the degenerative zone at the 10th to 15th day suggests that the degeneration is then at the maximum. Its persistence up to the 60th - 90th day suggests that some further destruction may occur up to that period. It is, therefore, probable that death of neural tissue following a 15-minute period of irradiation occurs to a 3 mm radius by the third day, extends to the 4 mm line by the 10th to the 15th day, and to the 5 mm line between the 60th and 90th day. The corresponding doses are 16,000, 6,000 and 2,000 rads. The late extension to the 90th day is not inconsiderable. The cross sectional areas of the early, intermediate and late stages are 28, 22, and 28 sq mm. The volumes of tissue involved are 260, 250, and 260 cu mm respectively.

Using these physical and biological measurements the needle has been used clinically to produce high cervical cordotomy, mesencephalic tractotomy, thalamotomy, pallidotomy, cingulotomy, trigeminal ganglionotomy and pituitary ablation. Isotopes such as palladium and thulium will probably prove more suitable for intracranial lesions of less than 7 mm diameter.

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PERCUTANEOUS CORDOTOMY FOR PAIN[*]

By

S. Mullan,[†] P. V. Harper, J. Hekmatpanah,[†] H. Torres,[†] and G. Dobben[‡]

Anterolateral cordotomy for pain, first performed in Philadelphia by Martin in 1912,[1] has a long established and honored position among neurosurgical operations. So undeniable are its merits that it has stood the test of time despite a mortality that has ranged between 4 and 25 per cent,[2] and a period of convalescence that is not inconsiderable. The lesion that is required in the anterolateral tract itself is very small, yet the operation is major. The lesion is of a magnitude that may be made accurately and discretely by the beta emitting radioactive isotopes, and it would seem that percutaneous introduction of such an isotope would be as effective as open operation with knife section. Such a needle puncture is impossible in the thoracic region because of the overlap of laminae and the posterior migration of the interarticular joints. The lack of overlap in the cervical region makes insertion lateral to the cord simple, and the anterior migration of the interarticular joints on C 1 and C 2 allows percutaneous access to the anterior as well as to the lateral aspect of the cord (Figs. 1 and 2). The vertebral artery enters
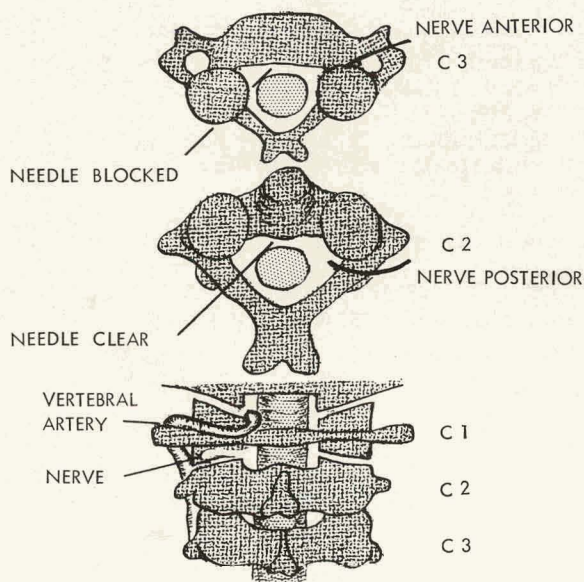

Figure 1. Top drawing illustrates how interpedicular joints interfere with anterolateral insertion of the needle in the lower cervical region. Bottom drawing illustrates unsuitability of the atlanto-occipital space because of the vertebral artery.

Figure 2. Air myelogram showing easy access to anterior surface of cord at the C 1 - C 2 interval. The position of the cord relative to the posterior surface of the dens varies with flexion and extension.

the dura through the atlanto-occipital space and the second cervical nerve enters through the C 1 - C 2 interval. The C 1 - C 2 interval is, therefore, preferable (Fig. 3).

Although those isotopes emitting beta particles of greatest penetration generally have the shortest half lives, it has been possible by using a strontium-yttrium source to provide both longevity of activity and penetration of particles. A strontium-yttrium needle suitable for our purposes has been constructed by one of us (P.V.H.).[3] Using the physical and biological data obtained in laboratory experiments, and knowing that the average cord at this level is 10 mm in a. p. diameter and almost 14 mm in width, it is possible to plan a field of beta irradiation to any desired depth in the anterolateral cord. Figure 4 shows such a field for a 15 minute irradiation. The needle, inserted at 45° to the sagittal plane, lies against the anterior dura 3 mm from the midline. Increasing or decreasing penetrations may be provided by moving the needle point in relation to the midline or by altering the period of radiation. A 5-minute dose would decrease the cut by 1 mm; a 30-minute dose would deepen it by 0.6 mm. Thus, duration provides a relatively fine adjustment. The position at the tip of a needle provides a coarse adjustment and is a most critical factor in the precision of the operation. Larger or smaller cords than average demand appropriate adjustment of the radiation field.

TECHNIC

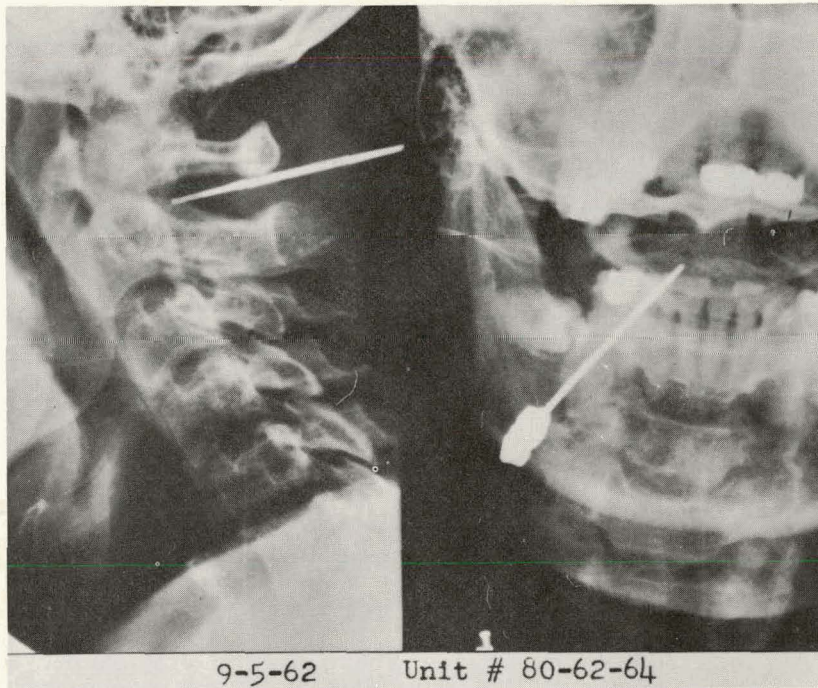Under local anesthesia a thin walled, No. 17, lumbar puncture needle is inserted between

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Figure 3. Radiograph of Sr^{90} Y^{90} needle in position for cordotomy.
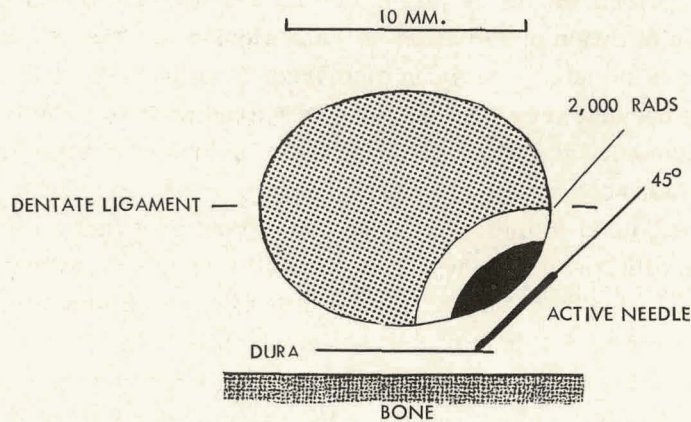
Figure 4. A needle entering at 45° with its tip 3 mm from the midline will deliver 16,000 rads at 3 mm from its axis (darkly shaded area), 6,000 rads at 4 mm and 2,000 rads at 5 mm if kept in place for 15 minutes. Death of tissue is complete in these zones on the 3rd, 12th and 90th days respectively. The cross sectional areas involved are approximately 5.25, 3.6, and 5.25 sq mm.

the 1st and 2nd cervical vertebra so as to approach the midline anterior to the cord at an angle of 45°. Local anesthesia must be perfect, for the patient will jump if the second cervical root is touched during insertion. We take about 10 minutes to carry out this stage of the procedure, using about 20 cc of 1 per cent procaine. The extradural space requires 1 cc of procaine. If not, the needle, having traversed the subdural space, will come up against the anterior dura from

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within and the patient may again jump. The No. 17 needle must be guided under suitable x-ray control. We prefer biplane television fluoroscopic image intensifiers, which throw anteroposterior and lateral images on two television screens in a fully lighted room. This equipment so facilitates the insertion that this stage may take no more than 1 minute to accomplish. The patient may remain prone, or lateral, or may sit up, depending upon his convenience and upon the limitations imposed by his pain. If a machine with only one plane is available, several rotations of the patient at right angles are required and insertion may take 10 minutes to accomplish. Standard x-ray equipment, using multiple Polaroid films is also very suitable. We have only once used standard equipment with standard films and this was very tedious, because insertion must be controlled millimeter by millimeter once the dura is reached. When the needle penetrates the dura, cerebrospinal fluid will be obtained. If cerebrospinal fluid ceases to flow, or if the patient experiences discomfort, the needle may be against the cord or a strong dentate ligament, and must be repositioned. Gently the needle is advanced until the anterior dura is reached. The sharp stilette is removed and an inactive replica of the strontium-yttrium needle is inserted. The No. 17 sheath is withdrawn to expose the terminal 6 mm of the replica, which remains in contact with the dura mater. As the No. 17 sheath is being withdrawn the elasticity of the muscles tends to pull it back toward the anterior dura. There is enough friction between the sheath and the replica to transmit this pull to the replica and keep it firmly against the anterior dura. (When the active needle is inserted it must not be allowed to slip posteriorly, for this will irradiate the pyramidal tract.) Careful measurements are made of the angle of insertion and of the position of the needle tip, which should lie just inside the shadow cast by the dens at 3 mm from the midline. The optimum duration of radiation is then calculated. This usually lies between 15 and 30 minutes, although 40 minutes have been employed. Long periods of irradiation to deepen the field are undesirable because they also lengthen it, extending it into the territory of the pyramidal tract. Central hemorrhages which resulted from lesions of greater than 30 minutes in the laboratory experiments, need not be a factor in cordotomy as the highest dose is expended in the cerebrospinal fluid lateral to the cord. When one is confident that the inactive needle is in optimum position it is withdrawn and the active needle is inserted. Protection of the operator from beta particles is provided by two small pieces of brass, which may be clipped onto the active point of the needle and to that part of the sheath which is held by hand. It is advisable to check the position of the active tip because a change of direction sometimes occurs during transference. When the needle has been withdrawn after the calculated period of exposure, the patient, if ambulatory, may walk off the table unaided. He is advised to lie flat for the next 24 hours, lest post "lumbar puncture" headache develop. One patient who went home the same day developed such a headache.

RESULTS

In all, we have performed 60 cordotomies upon 42 patients. The youngest of them was 3 years old, and the oldest 71. Thirty-seven suffered from malignant disease; five had benign conditions (Table 1). In 27 instances the operation was single unilateral; in five, two operations were done upon the same side. In eight the procedure was bilateral; one patient had three procedures, two being on the same side; one had four, three being on the same side. The results obtained are best understood by taking several examples:

Case 1. A woman of 41 years had known carcinoma of the uterine cervix for 2 years. She

Table 1

PATIENTS SUBJECTED TO PERCUTANEOUS CORDOTOMY

Carcinoma of Uterus	11
Carcinoma of Lung	9
Carcinoma of Prostate	3
Carcinoma of Breast	2
Carcinoma of Kidney	2
Carcinoma of Bladder	2
Carcinoma of Pancreas	1
Carcinoma of Thyroid	1
Carcinoma of Rectum	1
Neuroblastoma	1
Melanoma	1
Multiple Myeloma	2
Hodgkin's Disease	1
Diabetic Gangrene	1
Diabetic Neuropathy	1
Tabes	1
Herpes	1
Painful Paraplegia	1

had severe pain in her left leg and there was motor and sensory impairment of the anterior thigh. On August 1, 1962 a 30-minute cordotomy was performed, the tip of the needle lying 3 mm from the midline. Next day there was some improvement in her pain and toward evening of that day it completely disappeared. On the morning of August 3rd some sensory loss to pin prick was noted in all areas below L 1, and later that day the level was observed to ascend to T 8. Next morning it was at T 6. On August 11th it reached C 3, where it remained for the ensuing five months of her life. No further neurological deficit developed. Postmortem appearances of the cord are shown in Figure 5.

It is of interest that relief from pain was experienced before sensory loss could be determined by pin prick. The time interval may be much longer.

Case 2. A 58-year-old man presented in the Neurosurgical Clinic with "sciatica." It was obvious that he had lost weight and that his chronic cough had increased. He had carcinoma of the lung with multiple metastases. On August the 21st, 1962 a strontium-yttrium cordotomy of 25 minutes' duration was performed, the needle lying 4 mm from the midline. During the next week his sciatica gradually disappeared. Six weeks later pain relief persisted and there was no abnormal neurological sign. Ten weeks after the procedure there was partial pin prick loss to T 4 and at the end of 15 weeks there was complete loss to pin prick up to C 4. He was ambulatory during most of his remaining six months of life.

Sometimes pain relief, though very useful, is not complete in those patients who do not develop a sensory loss.

Case 3. A 51-year-old man had multiple myelomatosis for two years. There were multiple

Figure 5. Cord section at level of cordotomy. (Case 1)

deposits in his thoracic spine and he had severe left-sided chest pain for several months. On November 1, 1962 a strontium-yttrium cordotomy was performed, the tip of the needle being 5 mm from the midline and the duration of the cordotomy being 45 minutes. The next day he had considerable relief from pain and by the fourth postoperative day he stated that it had all disappeared. Moderately vigorous turning in bed could still hurt him, but although relief was incomplete it made life much more comfortable for this bed-ridden patient. Six weeks later he expired without having developed any sensory loss.

In addition to those patients for whom standard surgical cordotomy might be advised, this percutaneous procedure has been offered to several patients whose pain was insufficient to justify a major surgical operation, but enough to incapacitate them in their daily work.

Case 4. This 48-year-old woman, who had initial treatment for carcinoma of the cervix one year previously, had pain in her left leg for three months and limped considerably. A 22-1/2 minute cordotomy was performed, the needle lying 2 mm from the midline. Next day all her pain was gone. One month later when she returned for check-up, a sensory level to pin prick was present at T 5. She had not been aware of this change. Six months later she remains free from pain, and the sensory level is at C 4.

Percutaneous cordotomy may also be offered to patients too ill for consideration of a major surgical operation.

Case 5. This 48-year-old woman with terminal carcinoma of the cervix had known occlusion of one ureter. Oliguria developed with obstruction of the second ureter and her left-sided pain increased in severity. A 40-minute cordotomy was performed, the tip of the needle being in the midline. The next day all pain had disappeared. As she became increasingly drowsy, it became difficult to follow the exact development of her sensory loss and she expired 21 days later (Fig. 6).
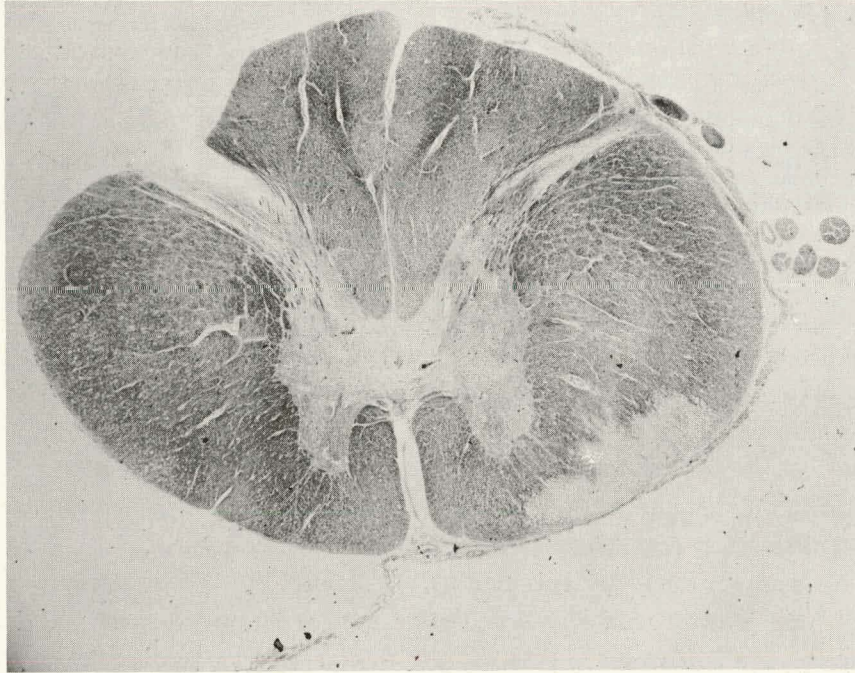
Figure 6. Cord section at level of cordotomy (Case 5). Note that swelling and degeneration extend beyond the area of necrosis.

We have been slow to use this new type of operation for patients who did not have malignant disease, but have employed it on five occasions.

Case 6. A 58-year-old diabetic had leg pain unrelieved by removal of his left L 5 - S 1 disc one year previously, in another hospital. His diabetes was under good control. There was bilateral stocking anesthesia but vibration sense was still detectable at the ankles. He had not worked for one year because of this pain. A 15-minute cordotomy with the tip of the needle 5 mm from the midline appeared at first ineffective. Two weeks after the procedure he reported a 5 to 10 per cent improvement in the pain in the left leg. After four weeks he reported a 60 per cent improvement. Seventy days later he reported that the pain in his foot had gradually disappeared and a sensory level to pin prick was present at T 8. At 100 days it stands at T 5.

COMPLICATIONS

Complications of insertion were less than we had expected. One nervous patient jumped in such a manner during insertion that the needle must have bruised or penetrated the cord. During the remainder of that day he had some weakness of fine movements in the corresponding arm and leg, but next day this weakness had entirely disappeared. The experience caused him to refuse any further attempt, and emphasized the necessity for adequate anesthetizing of the second cervical nerve. (As cordotomy was not performed, he is not included in results.)

Another patient during insertion complained of an "atomic explosion, a ball of fire." He saw it like a red flash and felt it hot on the right side of his head. Subsequently he had a proprioceptive loss in his right hand and a mild diminution to pin prick on his right face and right arm. It was supposed that the needle may have dislodged an embolus from a tortuous vertebral artery

and that this embolus lodged in his thalamus.

Perhaps most patients will complain slightly of a discomfort in the back of the neck during the following two or three days, but five have complained of severe pain in the back of the neck, which radiated up to the back of the head, and this has come on usually after several days and has persisted for 10 to 14 days. It is apparently due to irradiation in the occipital nerve and occurs even when there has been an absolutely painless insertion of the needle. A course of one to three injections of procaine at daily intervals or on alternate days have terminated or have coincided with the termination of this discomfort. Since the 2nd nerve lies close to the 1st vertebra, the needle should be kept close to the second.

Weakness. Weakness occurred in six instances, and was severe in one. Four patients were bed-ridden and in three the overdosage was quite deliberate, to ensure early relief of pain. Two were ambulatory and recognized significant but not incapacitating weakness in one leg. It is possible that other patients who were bed-ridden did develop weakness, but of a degree that could not be detected by routine neurological examination. One developed a positive Babinski response without gross weakness.

Sphincters. As many of these patients had pelvic disease, they were already wearing indwelling catheters. One of two patients who had bilateral cordotomy and who did not already have an indwelling catheter did develop paralysis of the sphincters on the fifth day after the second procedure. He had extensive Hodgkin's disease of the spine. No study has been made of the effects upon libido in any instance.

Unpleasant sensations. One patient with carcinoma of the prostate, who had pain down the back of his left leg, complained of a burning sensation in the same area forty-five days after the procedure. Soon afterwards, his objective sensory level began to appear. At the moment of writing (60 days after the cordotomy) he has dense analgesia below his knee, subtotal analgesia to the groin, and a very slight hypalgesic level at T 4. It is too early to say whether this is a temporary or permanent complication, as the level is still ascending. One other patient complained of occasional shock-like sensations down his spine during the period of developing analgesia.

FAILURES

Addiction. Three patients, who were taking massive doses of narcotics, continued to demand narcotics in spite of clinical and postmortem evidence of adequate destruction of the anterolateral tracts.

Case 7. This man, aged 51, had known carcinoma of the bladder for two years. For several months he had complained of pain in his back, sides and right shoulder. There was very extensive metastatic disease. On May 23, 1962 he had a cordotomy performed for his right-sided pain. This gave dramatic relief, but very soon he complained of equal pain on the other side. On August 23 he had cordotomy for this pain and it was repeated on September 6th. In spite of the development of adequate clinical and pathological evidence of anterolateral tract impairment, he continued to demand morphine every three hours.

Inadequate dosage. Of the five patients with benign disease, two (diabetic gangrene, diabetic neuritis (Case 6)), who had adequate cordotomy dosage got good results. Two (tabes, herpes) must be regarded as failures. In both the dose delivered was deliberately less than our theoretical calculations demanded. No sensory loss occurred and the failure could be either the result

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of inadequate dosage or resistance on the part of the disease. The patient with herpes had pain along the 7th intercostal segment on the right, and complained of intolerance to touch as well as of deep, burning pain. She developed relief of intolerance to touch only. It is difficult to evaluate the patient with painful paraplegia. The initial cordotomies of all seven patients who had two or more cordotomies on the same side must be regarded as failures due to inadequate dosage. All were corrected by the final procedure.

Anatomical abnormality. The patient who had four cordotomies falls into this category. Two cordotomies for right-sided pain (30 minutes at 3 mm from the midline) brought the sensory level to T 10 only. An identical cordotomy (30 minutes at 3 mm from the midline) on the other side produced a C 4 sensory within 3 days (which we would expect if the needle had been 1 mm from the midline). A third cordotomy (identical dose) for his right-sided pain produced a partial sensory loss up to T 2 within 3 days. Later it ascended to C 2 and he ceased to complain of pain though some islands of sensation remained towards the upper limit. It is postulated that his cord lies 2 mm to the right of the midline of his vertebrae. This may be due to his widespread metastasis from carcinoma of the kidney. (He seems too ill to advise myelography for confirmation.)

In short, effective cordotomies with relief of pain have been achieved in 34 of our 42 patients. Good objective sensory loss was obtained in three other patients who had become addicted to narcotics, but they still complained of pain. Two patients who had inadequate dosage did not obtain relief. One developed a burning sensation and feels no better. One is too difficult to evaluate and one is too early to evaluate.

DISCUSSION

This cordotomy, which basically attempts to duplicate the effects of a surgical cordotomy, differs in several essential ways. A most interesting one is the early development of pain relief before sensory loss becomes evident. This perhaps suggests that C fibers carrying dull pain are more susceptible to the radiation than are the myelinated fibers carrying pin prick. Neither temperature sensation nor tendo-Achillis pain was lost at this early stage.

The disassociation of pain loss and sensory loss seemed to be more evident in the case of pelvic nerve infiltration than in cases of destructive lesions in bone, though it was present in those patients also (Case 3). The delay of two or even three months in the full evolution of the lesion was anticipated from our experimental studies and it may be followed in Cases 2 and 7. This delay must be explained to those patients for whom minimal doses are employed. When maximal dosage is used, pain and sensory loss are complete within a few days, but motor loss may be expected in some of those patients whose survival exceeds three months. It is impossible to provide early relief of upper limb pain without risking this late motor loss, but a slower relief may be provided safely. Later models of the needle will have the isotope in the terminal 4 instead of the terminal 6 mm and this shortened field should eliminate damage to the pyramidal area. Up to the moment, we have not inserted the needle at an angle greater than 45° to the sagittal plane because of increased likelihood of irritation to the second cervical nerve, but it may be that a greater angle would be advisable in those patients in whom an early deep "cut" is desired.

Another point of interest relative to surgical cordotomy was that the sensory level has not been observed to fall in any patient, which is in striking contrast to our previous experience.

Sometimes islands of sensation persist in the upper limits, but these have not been observed to develop after analgesia had become complete. It is realized that the follow-up period is as yet very short.

One other observation was that in one instance pain did not disappear in spite of a good unilateral sensory level, but did disappear when a contralateral cordotomy was performed, suggesting that in some instances pain may ascend bilaterally. This suggestion may not be valid because since the cordotomy produces progressive destruction, it may be that this fortuitously became adequate at the period when the second cordotomy was carried out.

The advantages offered by this type of cordotomy are fairly clear. The elimination of mortality, and of the uncomfortable convalescent period are the most important. The technical simplicity is another. While it is true that the procedure was initially about as difficult as a myelogram for both patient and surgeon, it was soon evident that further experience on the part of the surgeon reduced this difficulty toward the magnitude of a lumbar puncture. It is now performed almost entirely by Dr. Hekmatpanah who is our second year resident. This technical simplicity and safety allow the surgeon to offer pain relief to a much greater number of patients than was possible with standard surgical cordotomy. It is possible to offer it to those who are too ill to survive surgical cordotomy, and to those whose life span is too short to make its inconveniences worthwhile. It is also applicable to those whose pain is too severe to be relieved by simple analgesics but not severe enough to demand a major operation. Lastly, those patients who have had a series of futile operative procedures for cancer and who, though otherwise suitable, are simply scared of another operation are usually very willing to accept the help offered by the simplicity of a nerve injection. It is certain that in any hospital population there are very many more patients who are suitable for this percutaneous method than for a surgical one. Allowing for the inevitable enthusiasm for a new procedure, it is significant that in a previous period one year ago, we performed less than ten surgical cordotomies. It is our feeling that the method bears about the same relationship to standard surgical cordotomy that percutaneous carotid arteriography bears to cut-down carotid arteriography. Modifications of the present needle and the use of other isotopes such as thulium remain to be explored.

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RADIATION THERAPY WITH HIGH-ENERGY ELECTRONS USING PENCIL BEAM SCANNING[*]

By

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In 1934 Brasche and Lange[1] reported in Strahlentherapie on the use of what they termed "fast cathode rays" as a method of therapy. This apparently is the first paper dealing with the direct use of an electron beam other than those from radioactive substances as a therapeutic agent. The authors seem to have been quite in agreement with those who have considered this modality more recently. They cite as advantages: (1) well-defined range; (2) ability to give off more energy at the depth than on the surface; (3) high biological activity; (4) magnetic directability. While they were talking about electrons with only superficial penetrability, they did produce epilation without superficial radiation effect using electrons ranging from 0.7 MEV to 2 MEV. No pigmentation was noted. They also noted that the effects of overdose were similar to those of x-radiation.

Trump and his co-workers described the therapeutic possibilities of electrons in their description of the Van de Graaff electrostatic generator in 1940.[2] This was, however, little improvement on the energy level of the original suggestion, although the generator was more elegant. Some clinical experience was presented, using what would now be termed "low energy electrons" by Trump in 1953.[3] There had been earlier reports from Europe of the use of electrons ranging from 1 to 6 MEV.[4-8]

The first mention of the use of high energy electron beam therapy was by Haas et al.[9] in 1954. In a paper dealing with electrons from a 22 MEV betatron these authors pointed out the advantages previously described and also stated that the skin reaction did not differ essentially from that experienced with other radiations. There have been a number of other reports in the literature. Uhlmann[10] describes the sharp cut-off in the mucositis produced in treating a tongue lesion through a single portal. He also describes mild skin reactions which cleared quite rapidly, and some of the advantages in treating deep-seated tumors by means of opposing portals. Lochman[11] has described the reactions obtained with a 19 MEV beam of electrons and mentions that the biological effectiveness is roughly two-thirds that of ordinary x-ray of 2 mm HVL Cu quality. As a consequence, the skin and tumor doses administered to his patients were considerably higher than those given to most others reported. He concludes that at this energy level there are important advantages in the use of electrons for treatment of subsurface and unilateral head and neck malignancies. Uhlmann[12] has used higher energy electrons, 28 to 33 MEV, for the treatment of pulmonary and esophageal lesions with promising early results.

Gale and Innes[13] have presented some very interesting dose distributions where mixed x-ray beams and electrons are produced simultaneously by the same machine, using specially designed targets. They show that this produces a flat beam and that the volume dose is consider-

ably lowered. Chu and her co-workers[14] have used electron beams of varying energy for the management of both inoperable and locally recurrent carcinoma of the breast with what is described as good palliative results in a high percentage of patients. In only 1 of 70 patients was there evidence of radiation pneumonitis, and skin reactions rarely progressed to small areas of moist desquamation, although tumor doses were in the order of 6,000 rads to most of the areas treated. In addition, they were able to retreat several patients who had had prior x-ray therapy.

Ovadia and McAllister,[15] who work with Uhlmann, have described the use of a grid in electron therapy to protect the skin and normal tissue overlying tumors. The need for this is somewhat surprising in view of Uhlmann's previously quoted statements which indicate minimal skin reactions. Ovadia states that the skin reactions observed normally do not limit the treatment but may be a source of discomfort to the patient.

Veraguth[16] has described clinical experiments using electrons from 10 to 30 MEV on 200 patients with a wide range of malignancies, some of whom had been previously treated by x-rays. He emphasizes the advantages of electrons, citing the uniform dose, the sparing of sensitive tissues near tumors, the reduced bone absorption and the skin sparing. He points out that the mucosal reaction differs but little from that produced by conventional treatment, but has observed a greater variation in time and intensity of reactions requiring closer observation of patients in order to judge response.

Zatz, von Essen and Kaplan[17] have used electrons from 10 to 40 MEV at Stanford. They state that while the electron beam has very little skin-sparing effect compared to mega-voltage x-rays, there is less skin reaction than with comparable doses of 200 KVP x-ray. Skin doses of 5,700 rads regularly produced severe dry reactions and usually wet reactions in large portions of the field, but the reactions were less for small fields. They agree with other observers that the mucosal reactions are similar to those produced by equal doses of x-ray both in time of appearance and intensity. While their patients were treated at a higher weekly dose rate and a fractionation schedule of three treatments per week rather than five, they do not feel that this is an adequate explanation for the increased skin effect. More recently, Perry et al.[18] have described in detail the methods of planning treatments for head and neck malignancies, using a range of 6 to 24 MEV. Smedal and his co-workers[19] have reported good results in 522 patients with a variety of superficial lesions treated with electrons of relatively low energy, 1 to 4 MEV.

Detailed discussion of the physical applications and dosimetry of electron beam therapy, has been adequately presented in the articles quoted above, as well as in many others. Only a brief description of the equipment will be made and some of the unusual applications which are possible will be mentioned. The device which has been used in these studies is the linear accelerator and pencil beam scanning system of the Argonne Cancer Research Hospital of The University of Chicago.[20] The accelerator is of the traveling wave type and is powered by two klystons in cascade (Fig. 1).

Energy control of the accelerated electron beam is obtained by setting the proper combination of radiofrequency power level of the klystrons and of phase of the electrons with respect to the radiofrequency wave in the second section of the wave guide. In this unit the electron energy is readily variable from 5 to 50 MEV.

On leaving the accelerator, the electrons enter the unique pencil beam deflecting and scanning system (Fig. 2).[21] The scanning device directs the electron beam, which is approximately

Figure 1. The 5 to 50 MEV electron linear accelerator of the Argonne Cancer Research Hospital. Three klystron stations are shown, one of them a spare. The accelerator tube proper is contained in the protective housing shown on the left side of the picture. (Photograph courtesy of High Voltage Engineering Corporation, Burlington, Massachusetts.)
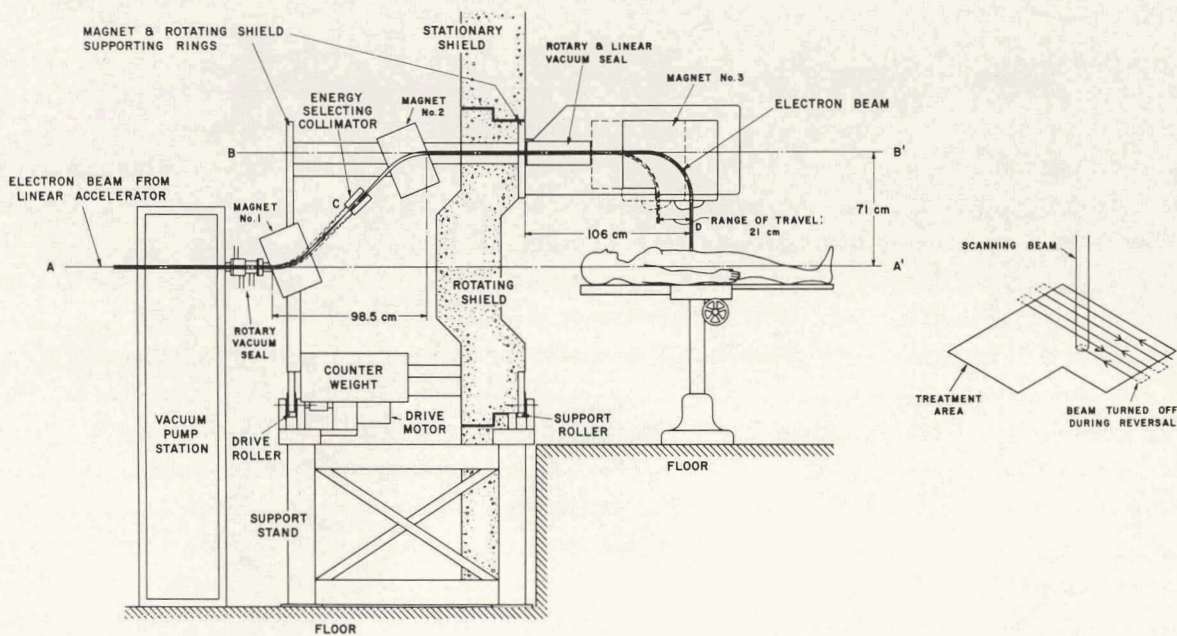

Figure 2. Schematic drawing of pencil beam deflecting and scanning unit showing arrangement of magnet system, counter weight, rotating shield, treatment cot and patient. The electron beam is contained within a vacuum chamber not shown in this sketch. Inset on right illustrates scanning over a single portal.

0.5 cm in diameter, over the tumor area of a patient. The beam is guided by means of magnetic fields and is capable of scanning arbitrary field sizes and shapes up to 20 x 20 cm under one treatment method (Figs. 3A and B, and 4). It can also be used to carry out convergent beam
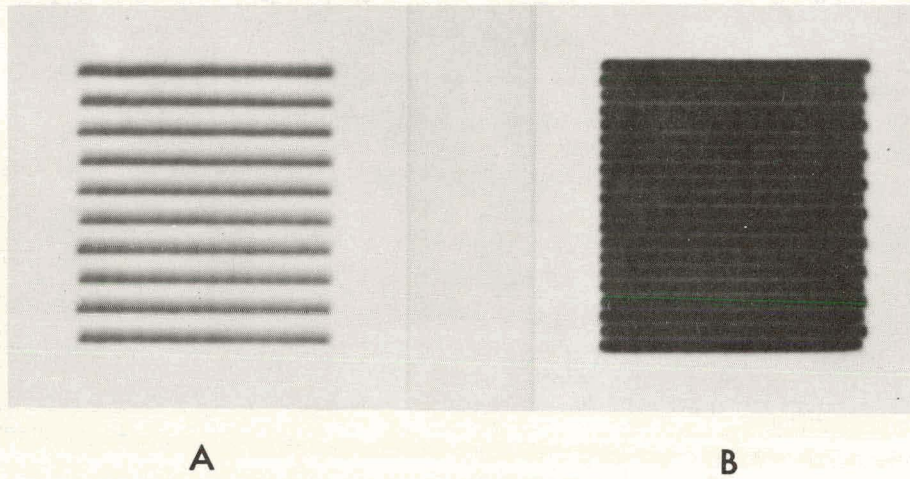

Figure 3. Scan pattern obtained by means of rotation and linear movement of magnet #3.
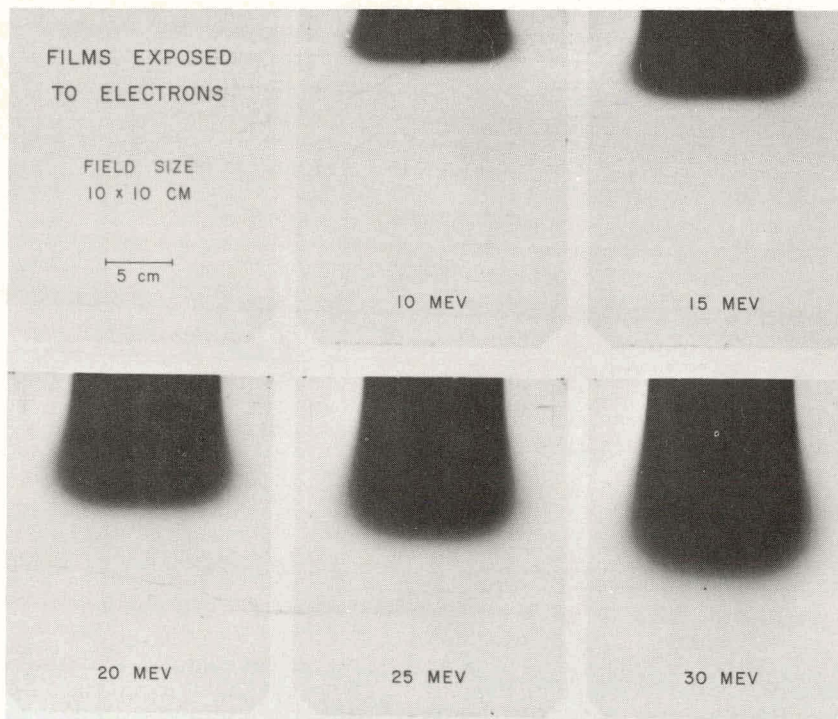A. Scan spacing 1.0 cm
B. Scan spacing 0.5 cm

Figure 4. Films, located within a phantom, exposed to electron beams of various energies.

therapy over an arc up to 360°, with a width up to 20 cm (Figs. 5 and 6). The beam is a pulsed beam with a repetition rate of 60 per second and a length of 1.2 microseconds. The patient area is elevated and a Siemens treatment cot is used (Fig. 7).
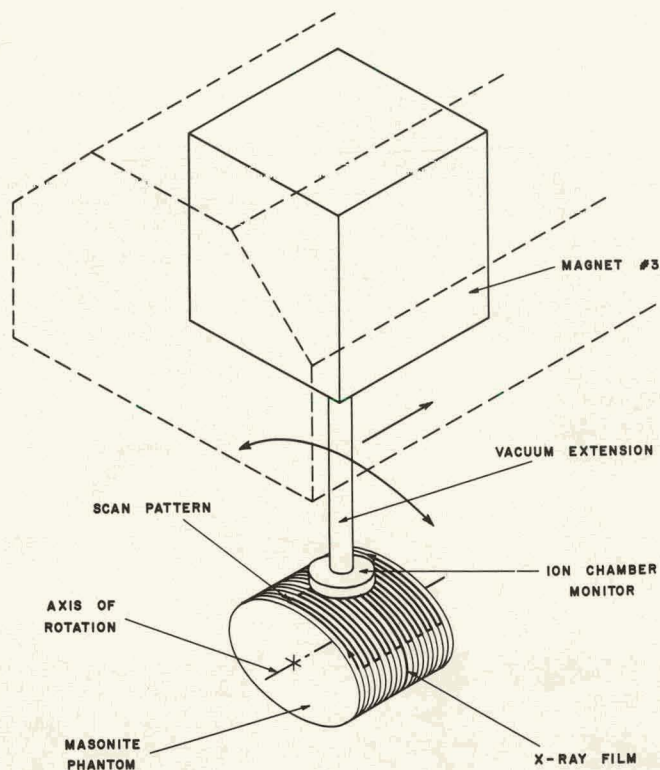

Figure 5. Schematic drawing of experimental arrangement for irradiation of phantom in field scan.
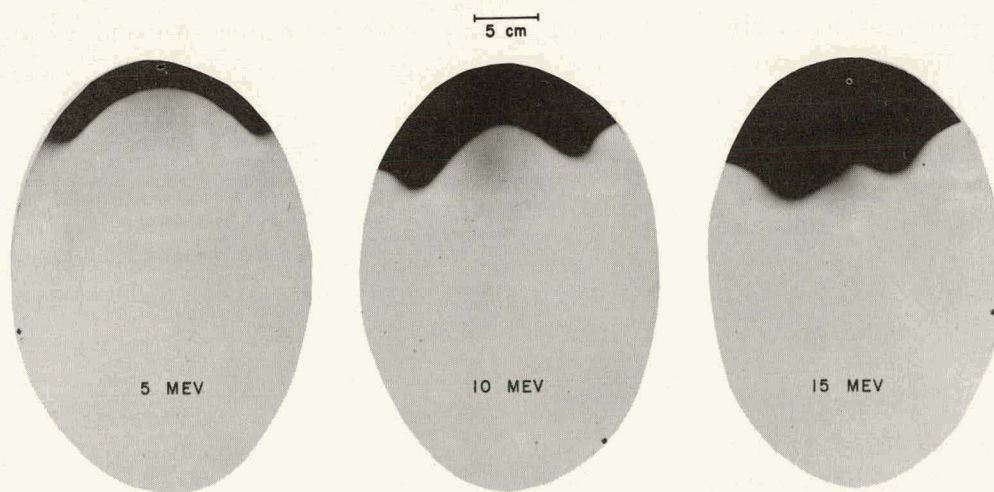
FILMS EXPOSED TO ELECTRONS

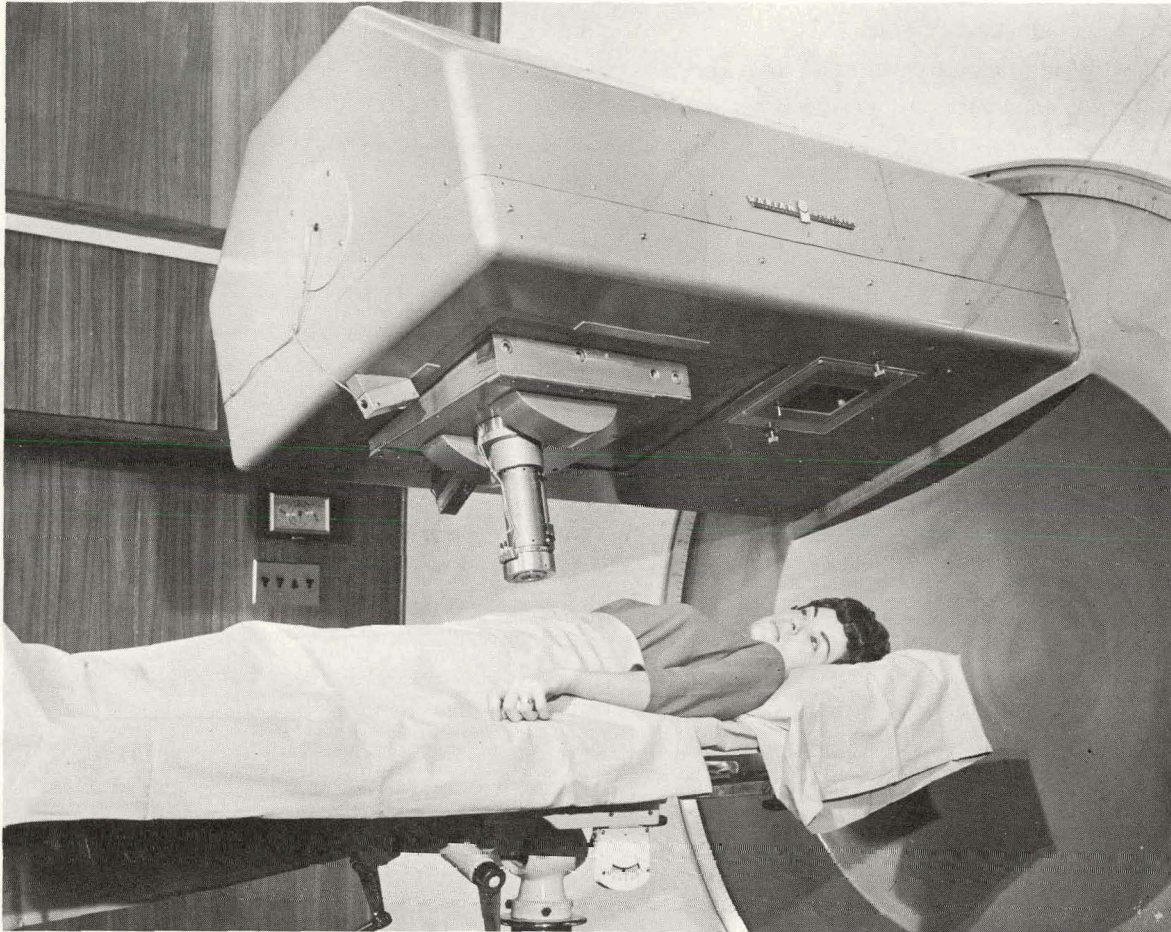Figure 6. Films exposed to arc scan beams of 5, 10 and 15 MEV.

Figure 7. Patient treatment area showing the final scanning magnet together with the vacuum chamber and transmission ionization chamber at the beam exit.

There are two quantities of the electron beam that need careful calibration to insure proper patient dosimetry:

(1) The energy of that portion of the beam which reaches the patient. The collimator delimits the electron energy to a band of $\pm 1/4$ per cent of the present energy. The energy of the beam is determined by magnetic deflection techniques.

(2) The intensity of the beam is established from a knowledge of the area covered by the beam per unit time, and the absolute value of the electron current. The electron current itself is measured by means of a Faraday Cage based on the design of Rozenfeld.[22]

The absorbed dose is determined on an absolute basis by making use of the energy and current intensity as well as the collision energy loss formulation of Sternheimer. This work on absorbed dose determination has been reported previously.[23]

The beam intensity is continuously monitored by means of a transmission ion chamber and the results are recorded (Fig. 7).

The very high dose rate of this system must be emphasized. If a typical cell of approximately 20 microns in diameter near the surface of a field which is receiving 300 rads in a single treatment is considered, it can be calculated that the cell receives its dose of 300 rads in 27 pulses of approximately one microsecond duration. The total time required to deliver these 27

pulses at 60 pulses per second is 0.45 second, and the average dose per pulse is 11 rads. The dose rate averaged over the one microsecond pulse is then 11 x 10^6 rads per second. In addition, the pulse is further modulated by the accelerating radiowave so that 2,856 bunches or groups of electrons arrive during each one microsecond pulse. The duration of each bunch of electrons is not known accurately with the sharp energy collimation used; it cannot be appreciably greater than 1/20th of the inter-bunch interval and it may easily be as short as 1/50th of the interval. The pulse average dose rate must be multiplied by the reciprocal of these factors to obtain the instantaneous dose rate. Therefore, the dose is delivered in pulses of about 0.004 rad each, with a dose rate of 200 to 500 times 10^6 rads per second. The other systems mentioned above all use foil scattering and, while the beams are pulsed, the dose rate is much lower than with the pencil scanning beam. It is possible that our high dose rate may play a part in the very limited skin reactions to be described later.

CLINICAL APPLICATION

As a preliminary to patient treatment, LD_{50} curves in mice were run with both high energy electrons and cobalt-60 gamma rays. The RBE determined from these runs was 1.00 ± 0.07 where the 0.07 represents the 19/20 confidence limit determination for the "potency ratio."

Since our first patient was treated on June 16, 1959, it is obvious that this presentation cannot deal with results of treatment in terms of cured disease. We have proceeded very slowly since we felt that we were dealing with an almost entirely new modality. Ninety-seven patients have been treated up to September 1962. Fifty-one had malignancies of the head and neck, 8 had intrathoracic tumors, 5 had mycosis fungoides, 1 had an extensive recurrent squamous cell carcinoma of the skin of the thorax, 4 had carcinoma of the urinary bladder, 3 had benign lesions, and the remaining 25 had a variety of lesions treated on an experimental basis, most of them for palliation only. Thirty patients received tumor doses of 6,000 rads or better through single portals, 15 had lower doses aimed at cure, either because of tumor sensitivity or because radiation was being used in combination with an experimental drug, and 42 patients received only palliative doses. In no case was a wet reaction observed and only in rare instances did a severe dry epidermitis develop, except when such reactions were deliberately produced by the addition of scattering material used to bring the biological dose of radiation up to the skin surface. In the parotid tumors and oropharyngeal lesions, the sharp cut-off of mucositis reported by others was most striking. Dry mouths with thick ropy saliva have not been noted, and we have altered our usual procedure of complete dental extractions in such patients and now remove only those teeth which would be included in the zone of radiation.

One of the first patients treated had a mixed tumor of the parotid. She had noted swelling and a hard mass in the right parotid for six months. We used a special lucite template, such as we make for all specially shaped portals, and delivered a tumor dose of 6,000 rads at 5 cm depth (Fig. 8). Twenty-eight treatments were given between July 6, 1959 and August 17, 1959. The energy of the electron beam was 14 MEV for the first ten treatments and 11.5 MEV for the subsequent ones. At the conclusion of therapy, the area of the single portal showed a moderate erythema which cleared rapidly. After three years there has been no evidence of recurrence and the skin appears normal except for a slightly coarsened texture (Fig. 9A).

The second patient to be described was first seen at our hospital in April of 1961, complaining of a growth on the upper gum. Several biopsies showed only leukoplakia, which was cauterized.
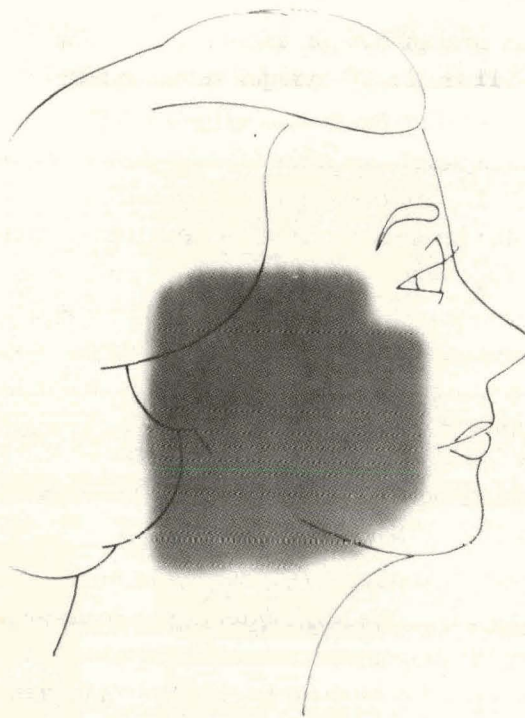
Figure 8. Scan pattern used for electron beam therapy on a particular patient.

The lesion grew in size, however, and another biopsy showed squamous cell carcinoma. When we saw her, there was a large fungating lesion involving the left upper gingiva, extending into the gingivo-buccal gutter, and having a plaque-like extension halfway across the palate. Using a single, 4.5 x 8 cm, portal and an electron beam of 20 MEV, we planned a tumor dose of 6,000 rads. Treatment started on December 6, 1961. After 20 treatments and a tumor dose of 4,000 rads, the skin showed a mild erythema. The erythema was brisk with some bronzing at the time of the 31st treatment, at the tissue dose of 6,800 rads (Fig. 9B). Thirty-two treatments were given with a tumor dose of 6,300 rads, completing the course on January 17, 1962. The maximum tissue dose was 7,050 rads. Within two and one-half weeks the skin reaction had almost entirely disappeared (Fig. 9C) and in 6 months the skin appeared normal (Fig. 9D).

Another patient had a history of sore throat of 3 months' duration when seen in January of 1962. A fungating lesion involving the right vallecula arytenoid, false cord and lateral pharyngeal wall was found. Biopsy showed squamous cell carcinoma in all areas. There was a large, very firm node palpable in the right carotid triangle. The patient had a past history of two myocardial infarctions. A tumor dose of 6,400 rads was given in 36 treatments between January 15, 1962 and February 27, 1962. The maximum tissue dose was 7,900 rads. The electron beam energy was 20 MEV except for the last 9 treatments, which were given at 18 MEV. The tumor dose was calculated at 8 cm, which was the 80 per cent level. One week before completion of treatment, there was a brisk erythema in the single 12 x 15 portal. At 2 weeks after treatment, the erythema was subsiding and in 6 months the skin appeared normal. The mucositis which appeared at 2,400 rads was severe at the end of therapy. The node decreased in size but still remains as a 1 cm hard nodule which has not changed in the last 5 months. There is edema of the

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right arytenoid but no evidence of tumor.

A patient with carcinoma of the tonsil was somewhat unusual. She had received 7,000 rads to the right tonsil for a squamous cell carcinoma in 33 treatments from September 28, 1959 to November 12, 1959. The radiation was given through a 180° sector of rotation, using our Co^{60} unit. In February of 1962 the patient was again referred for treatment, having developed a squamous cell lesion of the left tonsil. Because of the former irradiation, it was decided to use an electron beam in order to avoid the previously treated tissue as much as possible. An 8 x 11 cm portal was used at 13 MEV for the first 17 treatments and an 8 x 13 cm portal at 15 MEV for the last 7 treatments. A tumor dose of 0,450 rads was delivered between February 24, 1962 and March 31, 1962. The skin reaction at the completion of treatment consisted of a dry, scaling epidermitis (Fig. 9E) which subsided within a month (Fig. 9F). A maximum tissue dose of 6,800 r was delivered.

Quite a different problem was posed by a patient who was seen first in July of 1961, complaining of a mass in the nose and upper lip of 6 months duration (Fig. 9G). She had a long history of soreness in the inside of her nose and had been given three roentgen-ray treatments 2 years previously, and four more 1 year before. A biopsy showed squamous cell carcinoma. In this case we used 25 MEV electrons. In order to bring the maximum dose up to the surface of the lesion, a unit density wax mould was constructed. The patient received 35 treatments from August 2, 1961 to September 12, 1961 and the surface dose was 6,700 rads, with 5,560 rads at 9 cm depth from the mould surface. A wet reaction resulted (Fig. 9H) but 9 months later the skin appeared almost normal (Fig. 9I).

DISCUSSION

There appears to be considerable disagreement about the degree of skin reaction associated with electron treatment through single portals when the skin dose is in the neighborhood of 6,000 to 8,000 rads. Lochman[11] encountered wet skin reactions when the dose exceeded 7,500 rads. Zatz and his co-workers[17] have reported skin reactions comparable to ortho-voltage. There is also some disagreement as to the relative biological effectiveness (RBE) of electrons. Lochman and his co-workers conclude that the RBE is no greater than with roentgen-rays and probably less.

The observations described in this paper suggest that the skin reactions are less than those reported by most of the authors quoted. There are a number of possible explanations for this. Since the scanning beam traverses the area in a pattern similar to a television raster, the effect may be similar to treatment through a grid. However, the scanning is performed so as to give a practically homogeneous surface dose, particularly when day to day variations in port positioning are considered. Another possibility is that the pencil beam radiation is relatively clean compared with a foil-scattered beam. Finally, the actual dose rate to the skin must be taken into account. This dose rate is 200 to 500 x 10^6 rads per second. It may be that the latter part of the dose delivered to any cell is given during a period of relative anoxia, the bulk of the oxygen having been consumed during the first part of the time in which the dose is given. Dewey and Boag[24] found modification of the oxygen effect when bacteria are treated with large pulses of radiation as compared to unpulsed doses of the same amount given over relatively long periods of time.

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VISUALIZATION OF THE LIVER BY SCANNING USING Mo^{99} (MOLYBDATE) AS TRACER[*]

By

L. B. Sorensen and M. Archambault[†]

At present isotope scanning is the simplest and safest method for visualizing the configuration and structure of the liver. The basic principles of hepatic radioactivity surveys employing an automatic scintiscanner were outlined by Stirrett and associates.[1] The introduction of high-contrast photoscanning techniques[2,3] added greatly to the interpretation of liver scans. Tracers employed for scintillation scanning of the liver include radioactive colloidal gold (Au^{198}),[4-8] and a number of radio-iodinated compounds such as rose bengal,[6-10] tetraiodophenolphthalein,[6] iodipamide,[9] and human serum albumin.[11] Best results have been obtained with I^{131}-labeled rose bengal and colloidal Au^{198}. For a number of reasons, which will be discussed later, neither of these compounds is wholly ideal. It is the purpose of this report to describe the method and results of hepatoscanning employing radiomolybdate (Mo^{99}) as the tracer agent.

MATERIAL AND METHOD

Carrier-free Mo^{99} produced by thermal neutron fission of uranium oxide is available from Brookhaven National Laboratory as the ammonium molybdate. The radio-purity is better than 99.99 per cent (except for the daughter Tc^{99m}). Technicium-99m was obtained by milking from carrier-free parent Mo^{99} adsorbed on alumina. A special milking system and generator, from which Tc^{99m} can be milked repeatedly, is available from Brookhaven National Laboratory.

Radioassay of biological material for Mo^{99} and Tc^{99m} was done with a 3·3 inch well type sodium iodide crystal coupled to a transistorized 512-channel analyzer (Nuclear Data Model ND-130). External counting over the liver and head was done in the Argonne Cancer Research Hospital whole-body counting facility using 5·5 inch sodium iodide crystal detectors inserted into optimum-focusing collimators.

The usual dose of radiomolybdenum for scanning of the liver is between 40 and 50 microcuries, which is given intravenously in a single injection. Solutions for injections are sterilized by heat. Scanning is started 24 hours after injection, with the patient in the supine position. All scans included in this report were made with the commercially available Picker Magna-scanner[12] which uses a 3·2 inch thallium-activated iodide crystal, a 19-hole focusing collimator, a pulse-height selector, and two alternate data presentation systems, one of which is a background eliminating solenoid recorder, and the other a photographic recording system. In order to record the 0.140 MeV gamma radiation of Tc^{99m}, the lower and upper gate of the pulse-height analyzer are set at 90 and 200 KeV respectively. The scans are run at a speed of 40 cm per minute, with a distance between adjacent lines of the scan of 0.3 cm. Usually one hour is re-

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quired to perform a scan. Upon completion of the scan, the position of the liver edge, any palpable abdominal mass, and the costal margins are plotted on the scintigram.

   Biochemical and physical principles for use of Mo^{99} in liver scanning. A single tracer dose of Mo^{99} injected intravenously as sodium or ammonium molybdate disappears rapidly from the circulation of man (Fig. 1). Six hours after injection of carrier-free material into normal
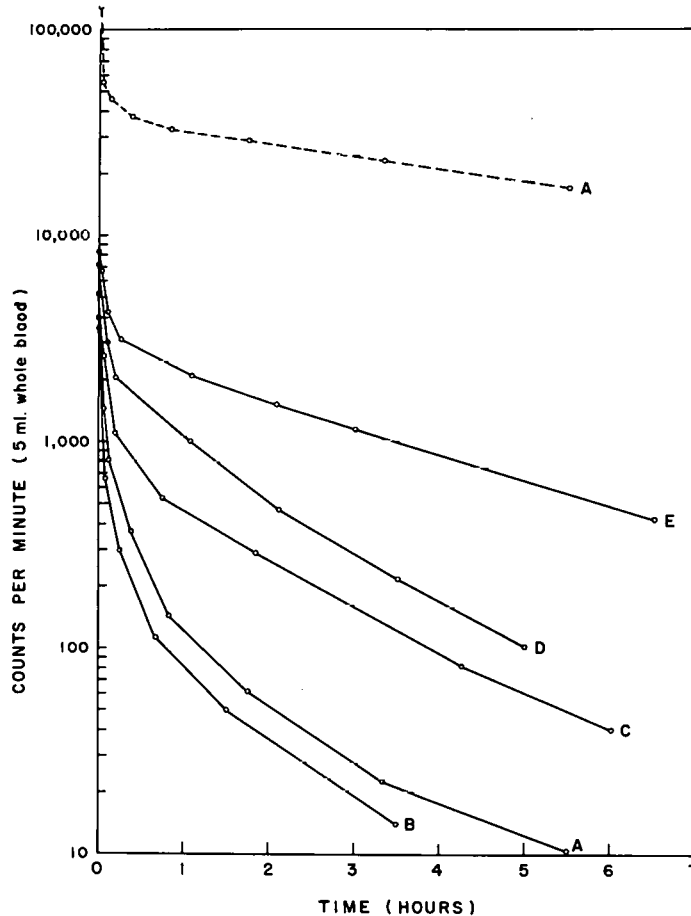

Figure 1. Disappearance from blood of Mo^{99} injected intravenously into four normal subjects (A-D) and one patient with viral hepatitis (E). Cases A and B received carrier-free material; cases C, D, and E were given 100 μg of Mo^{98} in addition to Mo^{99}. The stippled curve indicates disappearance of daughter Tc^{99m} in one of the experiments (corrected for decay).

subjects, the blood level of Mo^{99} falls to less than 1/300 of the initial concentration. When 100 micrograms of Mo^{98} is added as carrier, the disappearance curve is less steep, decreasing to about 1/100 after a similar lapse of time. Since only a minor portion of the administered molybdate is excreted during the first few hours, such rapid clearance signifies uptake of the element in one or more tissues. Secondarily the rate of clearance is dependent upon the diffusion space of molybdate, and the rate of excretion.

External counting (Fig. 2) as well as radioassay of tissues obtained postmortem, have shown molybdate to be selectively concentrated in the liver. From excretion data it is estimated that the uptake of Mo[99] by the normal liver is about 80 per cent when carrier-free material is injected. The biological half-life of Mo[99] determined by whole-body counting is about 20 days. Studies carried out in our laboratory have shown that labeled molybdenum is incorporated as a non-dialyzable component of xanthine oxidase in the rat. In man, this enzyme is located in the liver and possibly in the small intestine.[13] Detailed studies on the metabolism of molybdenum in man will be published in a later report.[14]
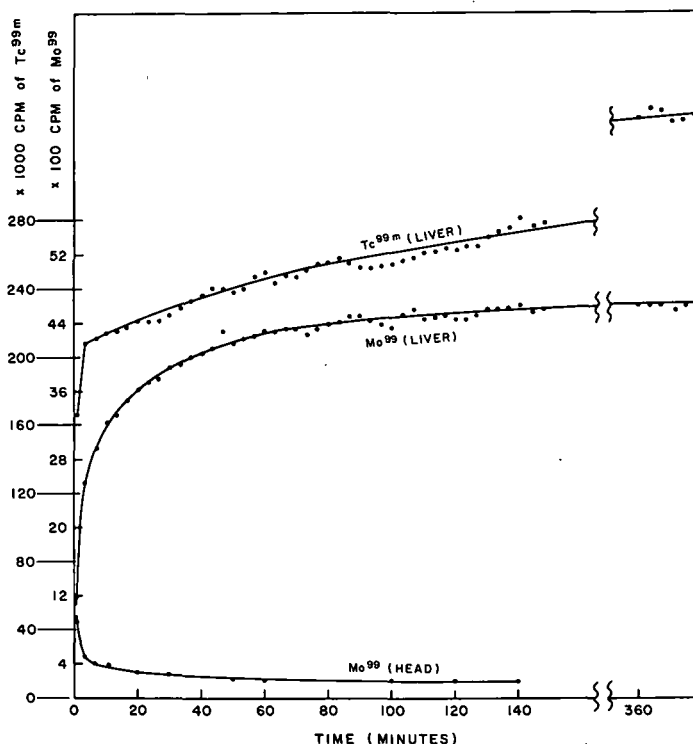

Figure 2. Measurement with collimated scintil-
lation probes of the rates of activity of Mo[99] and
Tc[99m] in liver and head following intravenous ad-
ministration of 40 μc of carrier-free Mo[99] to a
normal subject. (Not corrected for decay.)

Molybdenum-99 has a physical half-life of 67 hours; its decay scheme is presented in Figure 3.[15] Eighty-five per cent of Mo[99] decays via a 1.23 MeV beta to the 6 hour metastable technicium-99m; the remainder decays with emissions of beta 0.45 MeV (14 per cent) and 0.87 MeV (1 per cent), and 6 gamma emissions of 0.780, 0.740, 0.372, 0.181, 0.140, and 0.041 MeV. Two-thirds of this remainder goes directly to Tc[99], while one-third goes to Tc[99m]. In all, 90 per cent of Mo[99] will pass through Tc[99m] and thence to Tc[99] by isomeric transmission, emitting 0.142 MeV (1.6 per cent) and 0.002 + 0.140 MeV (98.4 per cent) gamma radiations. Technicium-99, with a half-life of $2.1 \cdot 10^5$ years, decays by emission of a 0.292 MeV beta to ruthenium-99, which is stable.

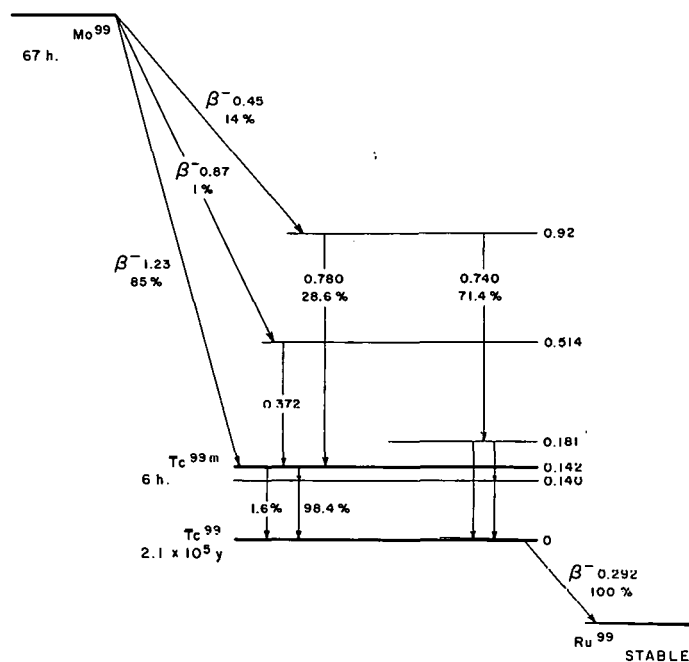The gamma radiation from Mo[99] itself is not suitable for scanning purposes, partly because

Figure 3. Decay scheme of Mo^{99}. The radiation utilized in liver scanning is the 0.140 MeV γ-ray of Tc^{99m}.

of its relatively high energy, and partly because of the low emission rate. However, a considerable increase in absolute count rate, as well as in contrast target—non-target ratio, is obtained by utilizing the low energy radiation of the daughter Tc^{99m}.

Technicium itself does not concentrate in the liver. This can be seen from Figure 4 which illustrates a parallel decrease in isotope concentration in the liver and head following intravenous administration of 100 μc of Tc^{99m} in the form of partechnetate. Six hours after its administration the level of Tc^{99m} in whole blood has fallen to 10.7 per cent of the initial value. Eighty-two per cent of the injected Tc^{99m} was recovered in urine and feces within 72 hours. Radioassay of excreta beyond this time was not feasible because of the short half-life of Tc^{99m}. On succeeding days the urine contained 47, 7 and 4 per cent of the administered technicium, while total recovery from feces during the same interval was 24 per cent. Whereas pertechnetate injected into the blood circulation does not concentrate in the liver, it is an interesting observation that Tc^{99m}, when produced from Mo^{99} already taken up by the liver cells, continues to stay in that organ. The liver may be likened to a column of resin which selectively retains molybdate, but not pertechnetate. The hepatic uptake of Mo^{99} is accompanied by a gradual build-up of Tc^{99m} (Fig. 2).

The growth of the daughter Tc^{99m} from pure Mo^{99} is illustrated in Figure 5. In this experiment, a mixture of the two isotopes was retained on a column of Dowex 1, chloride form (Ag-1-X-10; 200 - 400 mesh), 5 mm in diameter and 40 mm in length. When the level of molybdate solution had just reached the top of the resin, elution was begun with 1 N HCl to remove molybdate. The activities of Mo^{99} and Tc^{99m} in the eluate were determined as a function of time. Maximum count rate of Tc^{99m} was obtained after 23 hours, but equilibrium was not reached until 40 hours.

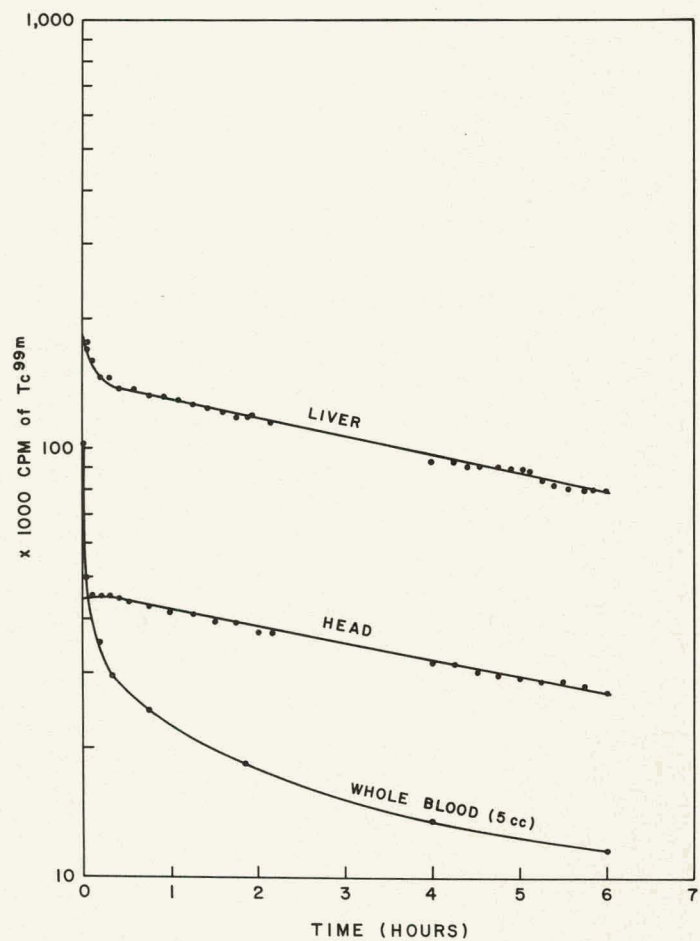A similar build-up of Tc^{99m} can be demonstrated in the case of the liver. Figure 6 shows

Figure 4. Decrease in concentration of Tc^{99m} in liver, head, and whole blood following intravenous administration of 100 μc carrier-free pertechnetate at zero time. (Corrected for decay.)
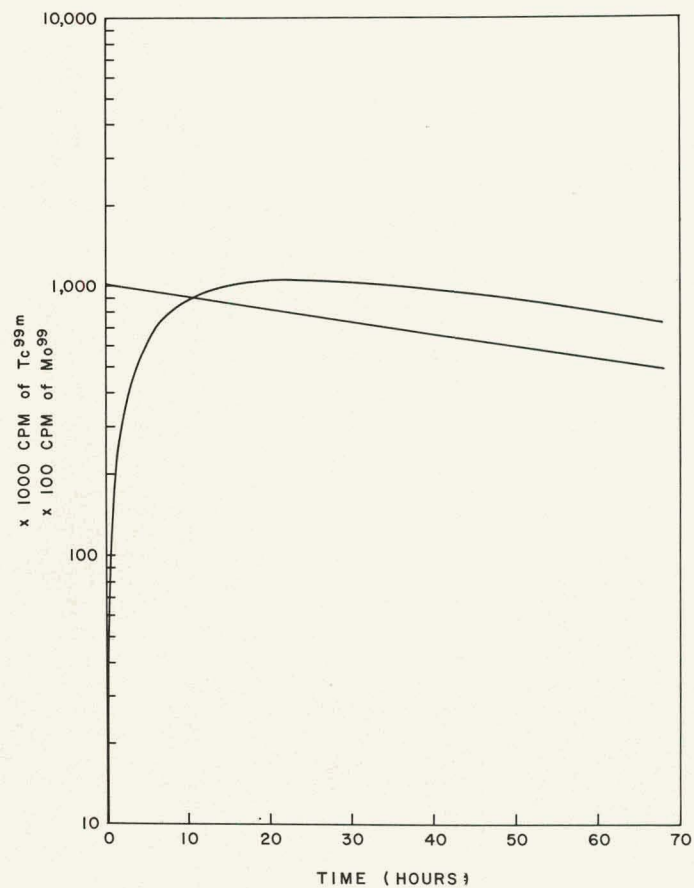

Figure 5. Activities of Mo^{99} and Tc^{99m}, showing growth of Tc^{99m} (rising curved line) from freshly milked Mo^{99} (straight line).
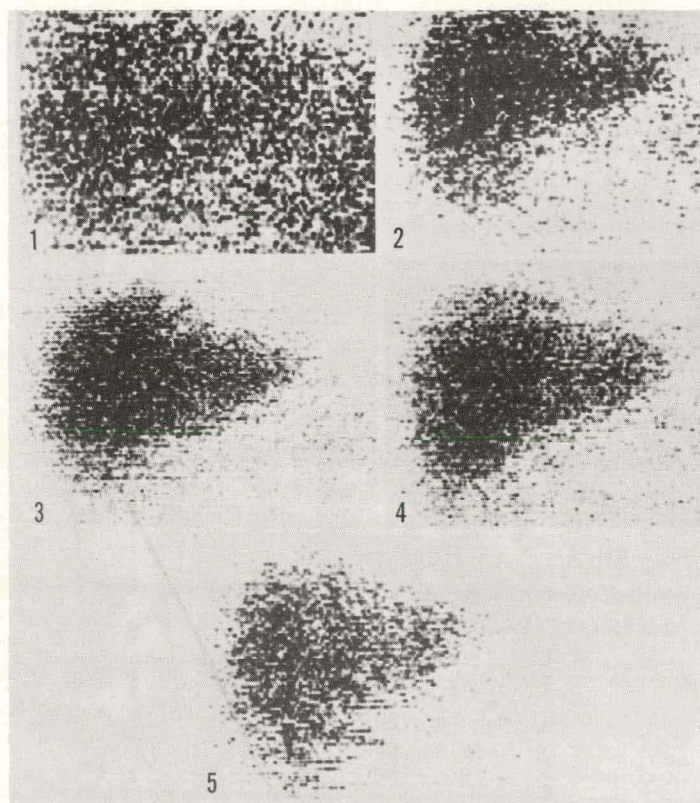
Figure 6. Photoscans of the liver done 3, 9, 21, 30, and 44 hours after intravenous administration of 40 μc of Mo^{99} to a normal subject.

five photoscans done 3, 9, 21, 30 and 44 hours after intravenous administration of 40 μc of Mo^{99} to a normal subject. The ratio between the maximum counts over the liver (counts per minute), and the background counts over the left inguinal region at the same times were: at 3 hours, 1500/1000; 9 hours, 2400/600; 21 hours, 4000/500; 30 hours, 3500/500; and 44 hours, 2800/400. Optimum scanning time is about 24 hours after injection of molybdenum. At this time, Tc^{99m} injected together with Mo^{99} has been almost lost, either by excretion or by decay, thus securing low background count; at the same time maximum build-up of Tc^{99m} has taken place in the liver.

Clinical reports. Approximately 100 liver scans using Mo^{99} as tracer have been performed in the University of Chicago Hospitals. Figures 7 through 12 are representative hepatoscans.

Neoplasmas, abscesses, and other space-occupying lesions destroy or displace the parenchyma resulting in loci of diminished or absent radioactivity.

In Figure 7 there is a huge defect along the lateral margin of the right liver lobe, with displacement of the functioning liver tissue downward and to the left. The patient was a 62-year-old woman of Greek origin who in 1947 underwent marsupialization and cauterization of an echinococcus cyst of the liver, and who presented with complaints of heaviness in the right hypochondrium and occasional pain of two months' duration. On the basis of the scan and the previous history, the patient was operated upon. At surgery, an echinococcus cyst 7 cm in diameter and multiple daughter cysts were found, involving the anterior aspect of the right lobe of the liver.

The 42-year-old man whose scan is shown in Figure 8 is known to have had bilateral poly-cystic renal disease with secondary hypertension for ten years. During the past two years he has noted progressive enlargement of the abdomen. Multiple filling defects are seen on the scan compatible with a diagnosis of polycystic hepatic disease.
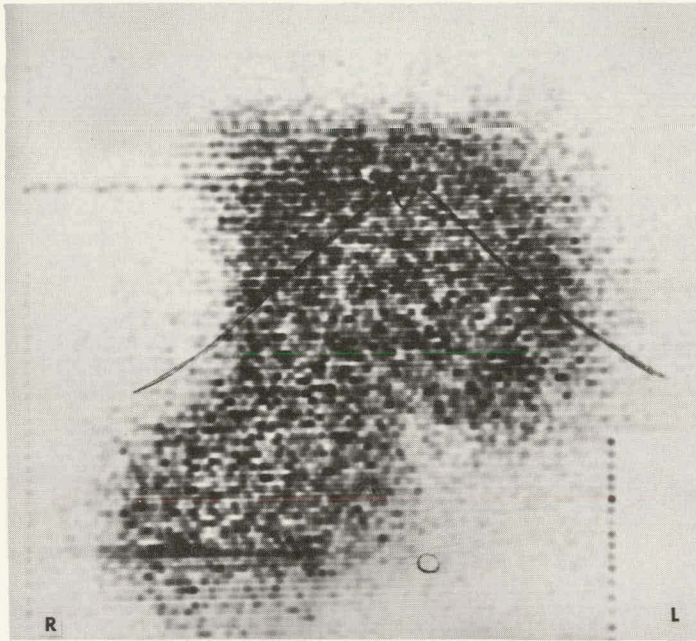
Figure 7. Photoscan showing large defect in the right upper quadrant of the liver proved at surgery to be caused by an echinococcus cyst.

Figure 8. Numerous filling defects throughout the liver, presumed to be cysts, in a patient known to have bilateral polycystic renal disease.
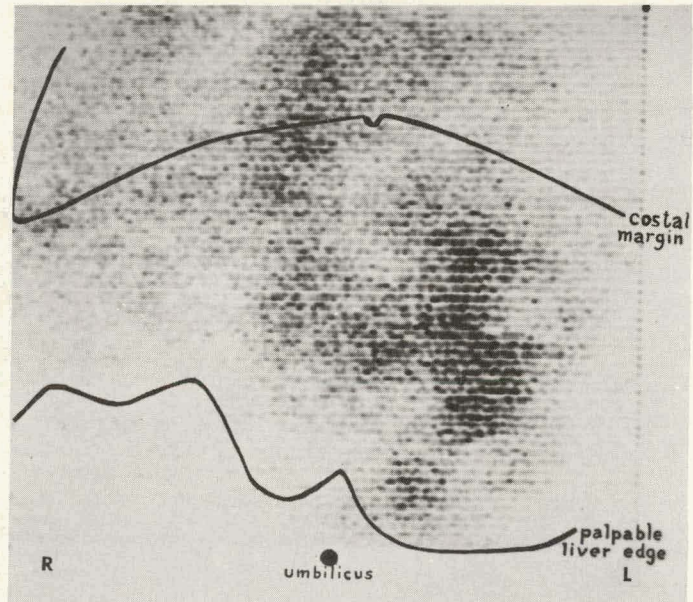
Figure 9 is a liver scan from a 46-year-old woman who was admitted to the hospital be-cause of abdominal pain and progressive jaundice of five weeks' duration. The hepatoscan shows a huge central filling defect, proven by postmortem examination 8 days later to be a metastatic carcinoma.

Figure 10 illustrates the scan of a 73-year-old man who was in good health until two months prior to admission. He presented with the finding of a firm mass in the epigastrium. The liver scan revealed a large solitary defect in the left liver lobe, proven by needle biopsy to be an undifferentiated adenocarcinoma of unknown primary origin.

Figure 9. Photoscan showing huge central defect, proved by postmortem examination to be a metastatic carcinoma.

Figure 10. Large lesion in the left lobe of the liver, proved by needle biopsy to be an undifferentiated adenocarcinoma of unknown primary origin.

Figure 11 is the scan of a 47-year-old man who entered the hospital because of progressive jaundice and epigastric distress of two weeks' duration. Two months prior to this admission, bronchial biopsy had revealed an 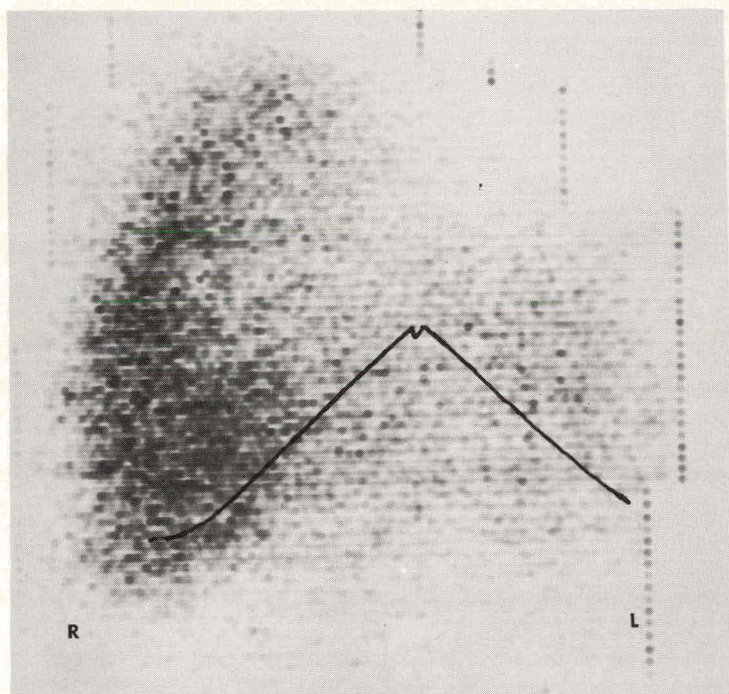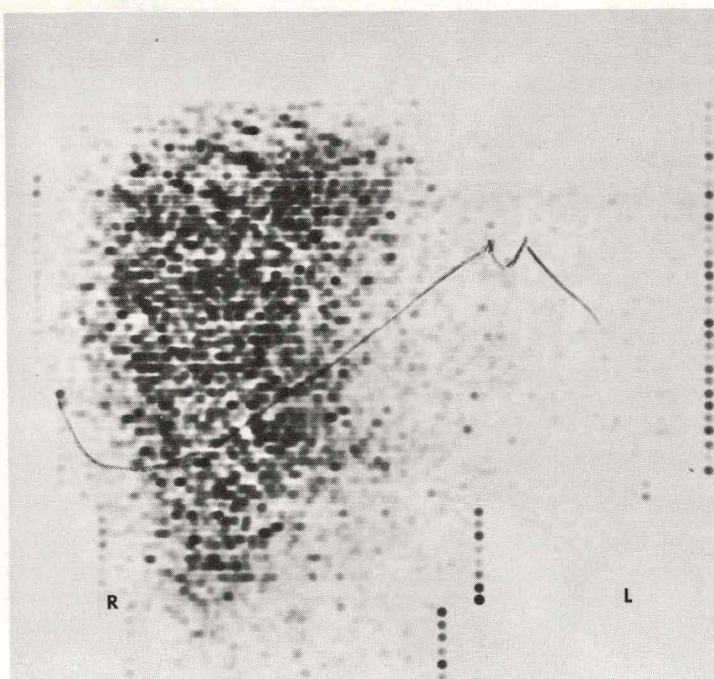undifferentiated small cell carcinoma as the cause of a superior vena cava syndrome. The scan shows several areas of decreased uptake indicative of metastatic disease.
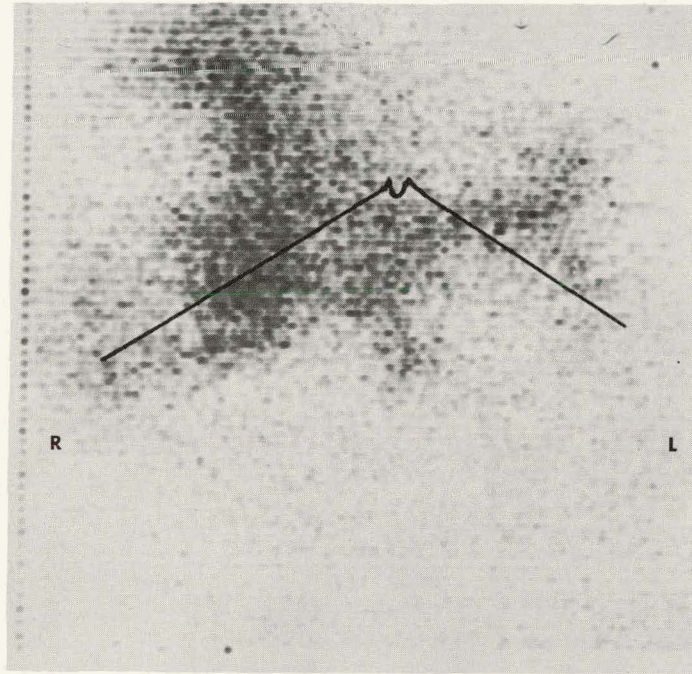
Figure 11. Photoscan showing several large filling defects caused by metastases from carcinoma of the lung.

No primary liver tumors have been encountered in this series; the possibility exists that a well differentiated hepatoma will select and retain Mo^{99}, thus producing a "hot" nodule.

In hepatocellular diseases, such as hepatitis and cirrhosis, the liver accumulates less of the administered dose of Mo^{99}, leaving more of the isotope available for urinary excretion. On the scan, the liver image appears reduced in density, with a pattern of patchy defects. The 59-year-old man whose scan is shown in Figure 12 was entirely well until 6 weeks prior to admission, when he noted the insidious onset of abdominal enlargement. Physical examination on admission showed a markedly distended abdomen due to ascites. The scan demonstrates a small liver with a mottled appearance suggesting multiple areas of fibrosis. Three weeks after the scanning examination, sudden massive gastrointestinal hemorrhage occurred. At postmortem examination 4 days after trans-thoracic ligation of esophageal varices, the liver was found to weigh 1000 g. The microscopic findings were compatible with a diagnosis of postnecrotic cirrhosis, with superimposed acute necrosis.

A similar mottled pattern of the liver scan can occur in disseminated metastatic involvement of the liver. Usually, the criteria obtained by conventional liver function tests and by liver biopsy are sufficient to formulate a correct diagnosis.

Figure 12. Small liver with numerous patchy
defects in a patient known to have postnecrotic
cirrhosis.

DISCUSSION

The clinical usefulness of radioisotope scanning of the liver is well established. From this
technique information can be derived about the size, shape, position, and internal configuration
of the liver. When the scan is superimposed over a thoraco-abdominal roentgenogram made with
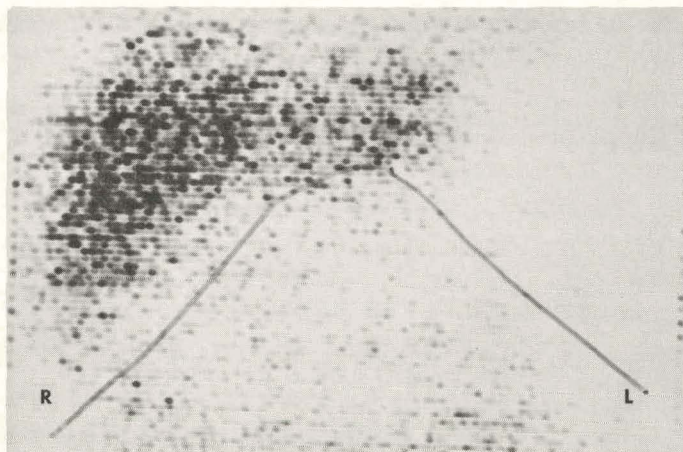the patient in the recumbent position, the relationship of the liver to adjacent structures can be
evaluated; this has proved especially useful in demonstrating liver displacement from an elevat-
ed right diaphragmatic leaf, as in subphrenic abscess.

Most studies of this kind have been done with I^{131}-labeled rose bengal or radiocolloidal gold
as tracers. Following its intravenous administration, rose bengal is selectively removed from
the blood by the polygonal cells of the liver and is rapidly excreted into the biliary system. As a
result of the short biological half-life, the concentration of I^{131} within the liver changes contin-
ually during the scanning procedure, which means that the initial setting of the scanning system
may not be the optimal one at the end. Furthermore, the release of rose bengal into the gall-
bladder and intestine may obscure the inferior margin of the liver. Colloidal radiogold is depos-
ited in the cells of the reticuloendothelial system which often remain unaffected in diffuse hepato-
cellular diseases; colloidal Au^{198}, therefore, is less effective in reflecting disease states which
affect the parenchyma. A disadvantage of radiogold is the high radiation exposure to the liver (8
- 15 rads).

Mo^{99} possesses a number of advantages over previously used tracers: 1) molybdate is se-
lectively concentrated in the polygonal cells of the liver. It has a long biological half-life, so
that the interval between the injection of isotope and the beginning of the scanning is not critical.
The scan can be repeated in case of technical failure without loss of quality; 2) the hepatic up-
take is closely related to the functional state of the parenchymal cells; 3) in comparison with
I^{131} and Au^{198}, the contrast ratio is considerably increased by the use of the low energy gam-
ma radiation of Tc^{99m}, since radiation originating deep in the liver beneath space-occupying
lesions will be largely attenuated before reaching the detector. The differential absorption ratio,
i.e., the amount of radioactivity over the liver to the amount of radioactivity deposited in imme-

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diately adjacent areas is between 6 and 10. With I^{131}-labeled rose bengal this ratio is generally between 2 and 4; 4) carrier-free Mo^{99} is in routine production by Brookhaven National Laboratory. The only preparatory measures involve dilution, sterilization, and radioassay, of an aliquot of the stock solution.

One probably slight disadvantage in the use of Mo^{99} is that one has to wait 24 hours before performing the scan. The possibility of detecting a lesion in the depth of the liver is reduced to a somewhat greater extent than with the penetrating radiation of I^{131}, but it is a matter of practical experience that such lesions, unless quite large, are much more difficult to detect by any type of scanning technique.

The radiation dose to the liver may be calculated using the formulae of Marinelli, Quimby and Hine.[16] Assuming a) an uptake by the liver of 75 per cent of an injected dose of 40 μc of Mo^{99}; b) no biological excretion; and c) complete physical decay, the total radiation dose to that organ would be slightly higher than two rads.[*]

The low energy of the principal radiation permits greatly reduced scintillation crystal size, and greatly enhances detector and collimator efficiency. A specially designed collimated scintillation detector system[17] which will increase the scan resolution by a factor of 2 is being tried.

Comparative studies of radiomolybdenum and materials hitherto in use for liver scanning are under way.

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[*]The amount of Tc^{99} which can maximally arise from 40 μc of Mo^{99} can be calculated to be $1.5 \cdot 10^{-6}$ microcuries.

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METASTATIC CARCINOMA OF PARATHYROID AND PERSISTENT HYPERPARATHYROIDISM*

By

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Carcinoma of the parathyroid gland is a well-documented but rare cause of hyperparathyroidism.[1,6-8] There are reports of nonfunctioning parathyroid carcinomas,[2,3] but the diagnosis is now reserved for functioning tumors of the parathyroid gland with distant metastases and/or dense adherence of the primary tumor to surrounding structures.[3,4] Local recurrence with invasion of local structures is probably an indication of malignancy.[10]

The differentiation of adenoma and carcinoma of the parathyroid gland by histologic examination is difficult. Atypical cellular arrangements, pleomorphism, bizarre nuclear forms, mitotic figures, prominent nucleoli, apparent invasion of the capsule and the presence of neoplastic cells within the lumina of blood vessels can be found in benign tumors,[4,9] while quite orderly tumors which are completely excised have given rise to distant metastases which become manifest later.

Six of the first 19 acceptable cases had an original histologic diagnosis of benign tumor.[2] It is difficult to make the diagnosis of parathyroid carcinoma from a metastatic lesion alone because of the resemblance to metastases from other endocrine organs and kidney.

Multiple endocrine tumors were reviewed by Underdahl et al.[5] who reviewed 14 cases and added 8 of their own. In these 22 cases the parathyroids were involved in 20. In these 20 cases, 11 had islet-cell tumors, and 3 of these 11 had duodenal ulcers.[5]

This report deals with a case of carcinoma of the parathyroid which was associated with hyperparathyroidism both before and after excision of the primary tumor.

CLINICAL HISTORY

The patient was a 61-year-old Negro laborer first seen at the University of Chicago Clinics in March, 1958, with complaints of joint difficulty for the past 5 months, inability to walk, weakness, and 24-pound weight loss. Laboratory examination revealed a marked anemia, elevated calcium and alkaline phosphatase, proteinuria, pyuria, and elevated BUN. He was admitted with a diagnosis of hyperparathyroidism. One year prior to admission here, he had been seen at a local community hospital, where, among other studies, the following were noted: NPN 34 mg per cent, serum calcium 16.0 mg per cent and serum phosphorus 2.6 mg per cent. During the subsequent 15 months he was readmitted 4 times for extensive metabolic studies. These will be reported in detail elsewhere and the following represents only a brief review of his clinical course prior to death.

During the first hospitalization (March-June, 1958) the laboratory findings of note included:

Hgb. 8.6 gm, serum calcium 15.4 mg per cent, serum phosphorus 2.9 mg per cent, alkaline phosphatase 20.7 units, BUN 21 mg per cent; x-ray examination showed bilateral kidney calcification, generalized osteoporosis, and bony destructive lesions characteristic of hyperparathyroidism. Of added interest was a significant reticulocytosis (5.8 per cent) and direct positive Coomb's test which together with his markedly depressed hemoglobin levels indicated an acquired hemolytic anemia of unknown etiology.

Surgical biopsy of a nodule palpated in the neck revealed tissue compatible with parathyroid adenoma or carcinoma. The patient subsequently underwent surgical exploration, and a 3.5 x 4 cm mass beneath the left lobe of the thyroid was removed. A lymph node, at the superior left lobe of the thyroid, removed with the mass, contained tumor similar to the main mass indicating metastatic spread of a parathyroid carcinoma.

Postoperatively there were no significant alterations in the serum calcium, phosphorus, or alkaline phosphatase abnormalities. However, tubular reabsorption of phosphorus which had been 49 per cent preoperatively increased to 72 per cent postoperatively, indicating some decrease in parathyroid hormone activity. Symptomatically, the patient improved considerably and was discharged on the 65th hospital day with a diagnosis of persistent hyperparathyroidism due to metastatic parathyroid carcinoma.

During 4 subsequent hospitalizations, the patient underwent extensive metabolic experiments in which efforts were made to control the growth of the metastatic tumor and the severe chemical derangements of persistent hyperparathyroidism. Included in this course of therapy and study were attempted hypophysectomy utilizing yttrium (Y^{90}) pellet implantation into the pituitary, prolonged dietary management, and administration of anabolic hormones (testosterone and growth hormone). These experiments will be reported in detail elsewhere.

His final admission was in June, 1959, because of recurrent generalized bone pain. Laboratory examination revealed marked anemia, calcium 8.0 mg per cent, phosphorus 3.0 mg per cent, and alkaline phosphatase 8.3 units. X-ray examination revealed progression of bone lesions of hyperparathyroidism with considerable vascular calcification. Because of reported tarry stools immediately before admission, he was transfused with whole blood and received active ulcer management. He appeared to be comfortable and improving medically, when he suddenly died 12 days after admission.

AUTOPSY FINDINGS

External examination revealed a cachectic Negro male with old healed surgical incisions in the cervical and inguinal regions, cyanosis of the nailbeds, and asymmetry of the chest due to multiple fractures of the ribs. The upper third of the right femur and the left clavicle had mobile fractures. There was generalized osteoporosis, and the marrow cavities of the skull, clavicles, ribs, iliac crests, and sternum were partially replaced by a firm light-gray tissue. The marrow cavities of the vertebrae, femora, and tibiae were partially replaced by a red-brown semisolid material. Microscopic examination confirmed the generalized osteoporosis; the marrow in all areas examined revealed cystic fibrosis; there was replacement by a loose, myxomatous connective tissue in which there were small islands of fat, hematopoietic tissue, and cartilage.

The only remaining parathyroid tissue was a 10 x 5 x 5 mm soft tan gland situated on the right upper pole of the thyroid. On microscopic examination the gland was approximately 30 per cent fat; however, the fat was not evenly distributed throughout the gland. One pole of the gland

was relatively free of fat (Fig. 1). Water-clear and intermediate cells accounted for about 80 per cent of the cells.

Metastatic carcinoma was found in 4 hard, lower left cervical nodules which were densely adherent to surrounding tissues (Fig. 2). Each was 5 mm in greatest diameter. Other cervical, mediastinal, and abdominal lymph nodes were free of tumor. There were numerous smooth, light-gray tumor nodules, up to 10 mm in diameter, evenly distributed throughout both lungs. On microscopic examination (Fig. 3), these metastatic nodules resembled those in the cervical lymph nodes. In the right lobe of the liver there was a 30 mm spherical, well-demarcated, gray, partially cystic metastatic nodule (Fig. 4) which on microscopic examination had less stroma and a more organoid architecture than the other metastatic tumor (Fig. 5).

The pancreas was 190 g, firm, the usual shape, and on microscopic examination contained, in the body, an islet-cell adenoma (Fig. 6).

In the gastrointestinal tract there was a 15 x 15 mm sharply demarcated ulcer in the first
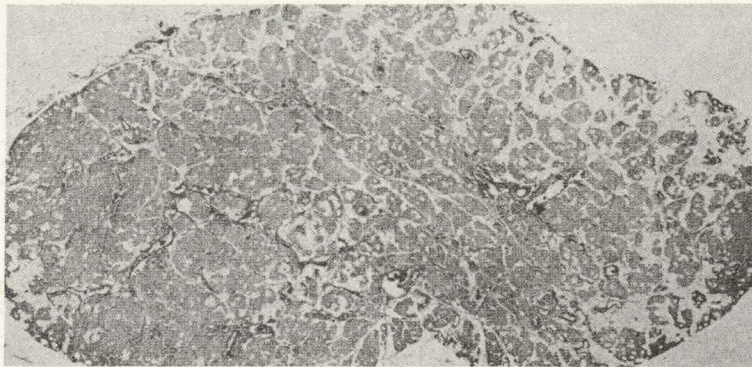
Figure 1.   Remaining parathyroid gland at autopsy.
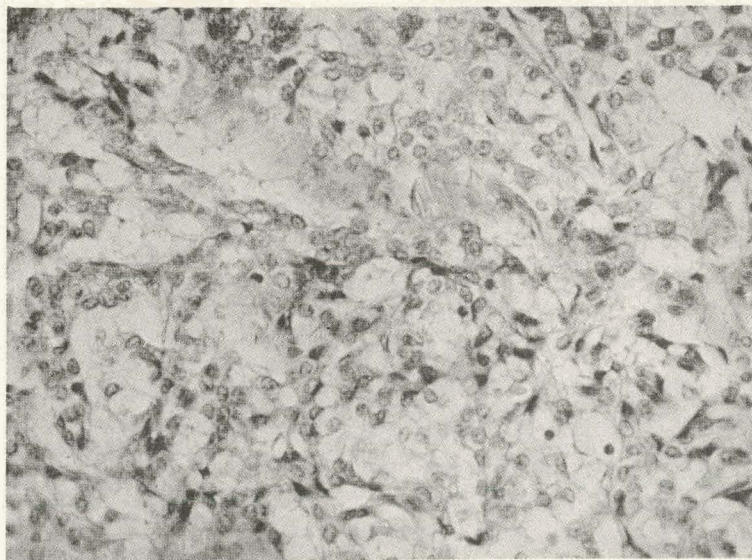
Figure 2. Metastatic carcinoma from the parathyroid in cervical lymph nodes.
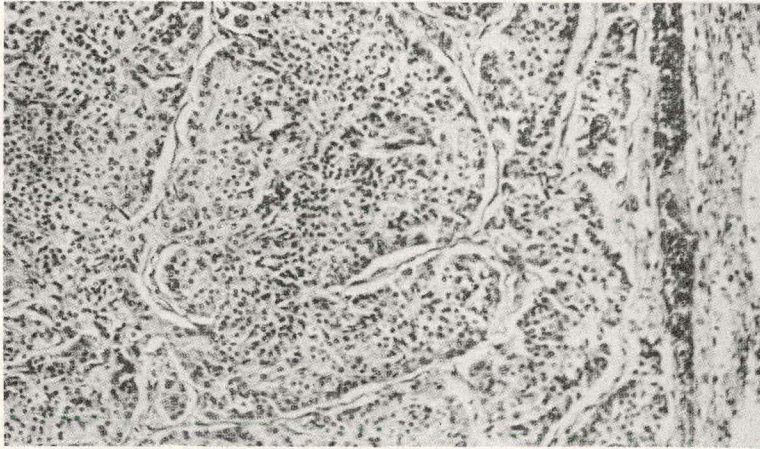
Figure 3. Metastatic carcinoma from the parathyroid in lung.

Figure 4. Liver with metastatic carcinoma from the parathyroid.
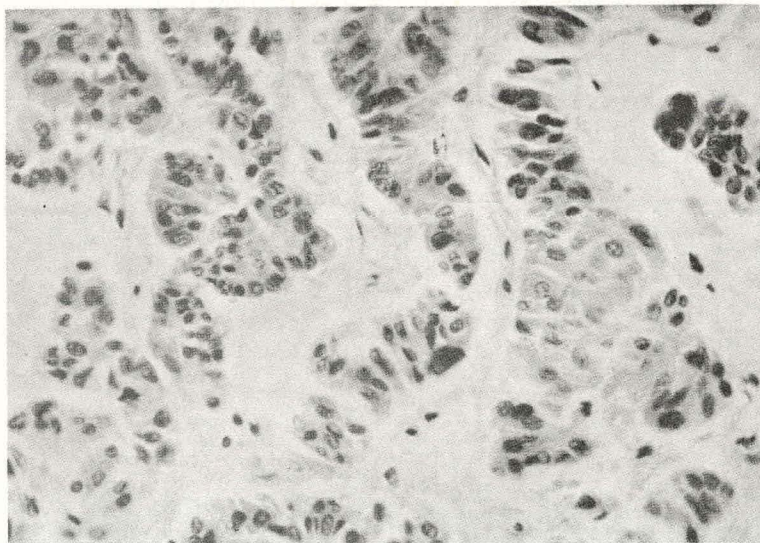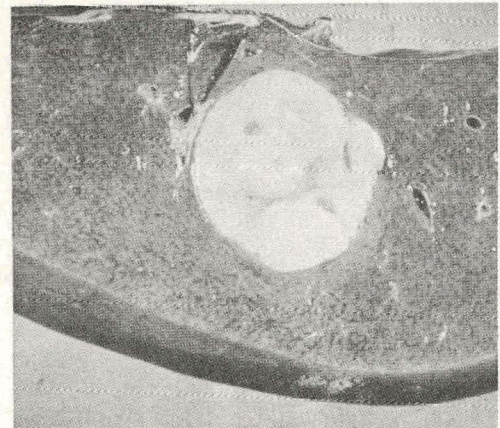
Figure 5. Metastatic carcinoma of the parathyroid in the liver.

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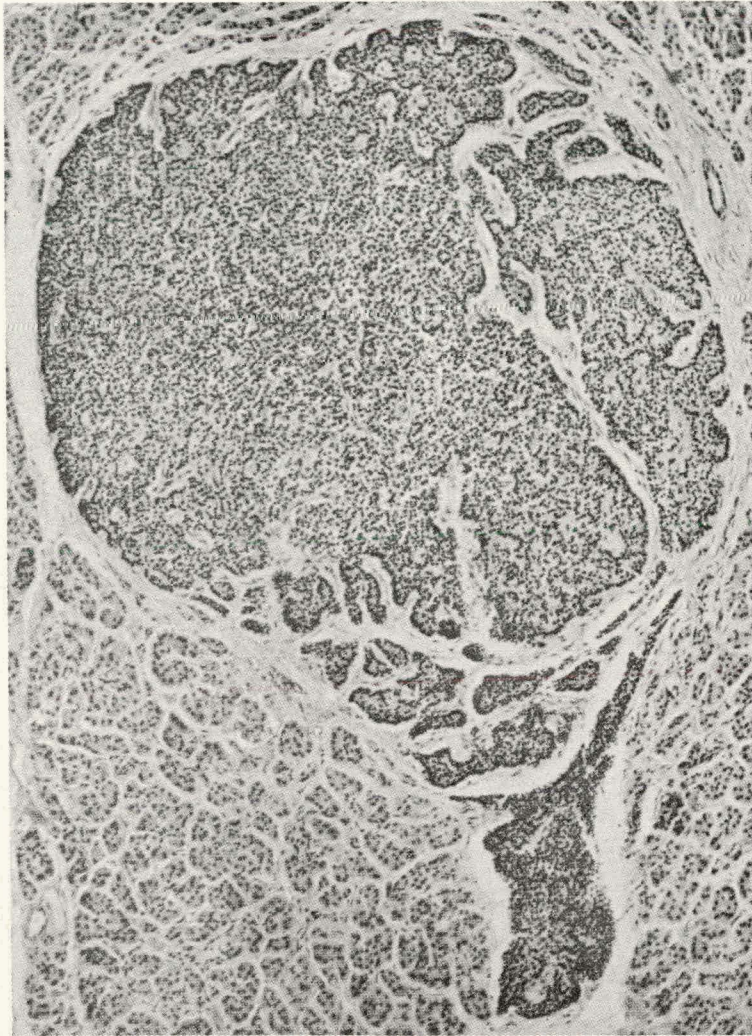
Figure 6. Islet-cell adenoma of the pancreas.

portion of the duodenum. On microscopic examination the ulceration extended only to the sub-mucosa and had a base of granulation tissue. There was also a superficial gastritis of the stomach.

Other findings directly related to the parathyroid carcinoma included, in the respiratory tract, extensive calcification of the alveolar septae, calcification of the tracheal and bronchial cartilages, and calcification of the basement membrane and submucosal glands of the trachea. In the genitourinary tract there was a 20 x 5 x 5 mm yellow, irregular calculus in the urinary bladder, calcified material in the papillary tips and calyces, and, on microscopic examination, extensive calcification of the tubules of the cortex and medulla. Calcium was deposited in the epididymus. On microscopic examination of the cardiovascular system there was focal calcification of the myocardium and extensive medial calcification of systemic arterioles. The osseous system was described above. Focal calcification was present in the greatly involuted thymus.

Other findings included: edema of the larynx without serious compromise of the airway, pul-

monary edema, mild chronic tracheobronchitis, and focal bronchopneumonia. There was mild chronic pyelonephritis and a partially calcified cortical adenoma in the kidney. There was a left hydrocele and atrophy of the testes. Fibrosis in the myocardium and a moderate degree of atheromatous change, partially calcified, in the anterior descending and left coronary arteries as well as the abdominal aorta were present. The thoracic aorta was less involved and the right coronary, renal arteries, and circle of Willis were free of disease. The adrenals were 5 g each and on microscopic examination were lipid rich and contained a few small myelolipomata. The spleen was normal on gross and microscopic examination.

The pituitary gland was serially sectioned; approximately 15 per cent of the anterior lobe remained intact as a thin sheet of tissue just under the stalk, the remainder consisted of amorphous debris and coagulation necrosis of cells. The general shape of the gland and its surrounding bone was normal.

The central nervous system revealed no gross or microscopic abnormalities.

COMMENT

In the first 24 cases of carcinoma of the parathyroid[3] the metastases fell into only 1 of the following categories for each case: (1) visceral metastases, (2) lymph node metastases, (3) local recurrence, (4) local invasion without recurrence. This case differs due to the fact that there were both visceral and lymph node metastases.

The other unusual feature in this case is the persistent hyperparathyroidism after removal of the primary tumor and in the absence of local recurrence. Even though the remaining parathyroid gland was enlarged it did not contain an adenoma, and we feel that it is less likely that this was the source of the hormone than the metastases.

A complete failure of either subjective or objective improvement after hypophysectomy lends further evidence to the independence of the parathyroid gland from the pituitary.

Because of the extensive destruction of the pituitary gland it was not possible to determine if this organ participated in the multiple endocrine tumors in this case.

SUMMARY

A case of carcinoma of the parathyroid is described. The hyperparathyroidism persisted after removal of the primary tumor and in the absence of local recurrence. The usual sequelae to prolonged hyperparathyroidism were present; in addition there was an islet-cell adenoma and duodenal ulcer. The metastatic pattern differs from other reported cases.

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FEVER-PRODUCING ACTION OF STEROIDS[*]

By

R. H. Palmer and A. Kappas

INTRODUCTION: PROGESTERONE THERMOGENESIS

The relation between ovarian activity and the temperature alterations which accompany pregnancy and the menstrual cycle has been recognized since the early part of the nineteenth century. The general features of this relationship are well known and need only brief mention.[1-4] Basal body temperature falls during, or slightly preceding the menstrual flow, remaining depressed until the mid period; at or near the time of ovulation body temperature rises, remains elevated during the luteal phase of the cycle and then falls again with the succeeding menstrual flow. During the first few months of pregnancy a similar increase in basal body temperature occurs but this subsides as gestation proceeds. It is generally accepted that secretion of progesterone by the ovary accounts for these temperature elevations, though this effect of the luteal hormone can be modified by concurrent secretion, or exogenous administration, of estrogen.[5-7]

The thermogenic activity of progesterone has been thoroughly confirmed and this hormone appears to be the first steroid shown to have temperature-elevating properties in man. Intramuscular administration of this compound in small amounts (10 to 15 mg per day) consistently induces small but significant temperature increases in human beings and in several species of experimental animals.[7-9] These increases, which rarely exceed 1 to 1.5° F, develop several hours after administration of the hormone and after single injections may last for 48 or more hours. In animals, prolonged latent periods are noted unless the hormone is administered intraperitoneally.[9] Repeated injection of progesterone in man leads to sustained though small elevations in basal body temperature; these subside shortly after administration ceases. Progesterone and several synthetic progestational agents are thermogenic when given by mouth also,[10,11] but considerably larger amounts of steroid are required for this effect than when the hormones are injected intramuscularly. The mechanism of progesterone thermogenesis has not been examined in detail and the significance of this action has not been considered to extend clinically beyond its role as an indicator of normal ovarian activity.

PYROGENIC STEROID METABOLITES

Recently a new class of steroids having strikingly more powerful thermogenic properties than progesterone has been described, and it appears that these compounds may be involved in some way in the pathogenesis of certain febrile clinical disorders in man.[12-16] The structural prototype for this new class of substances is the steroid metabolite, etiocholanolone, a 17-keto-steroid derivative of testosterone and other gonadal and adrenal hormones.[15,17] Intramuscular injection of this and related steroid pyrogens in man induces a fever which begins after a variable latent period of four to eight hours, reaches a peak in approximately 12 hours and generally subsides within 24 hours; occasionally smaller temperature elevations persist or recur for

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[*]This paper appears in The Medical Clinics of North America, Philadelphia: W. B. Saunders Company, 1963, p. 101.

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two or three days after intense reactions. Febrile responses are generally proportional to the dose of steroid injected, and may exceed 6° F. Polymorphonuclear leukocytosis accompanies the fever and marked increases in metabolism appropriate to the rise in temperature are observed (Figs. 1 and 2). Temperature elevations are reproducible and consistent and there is no
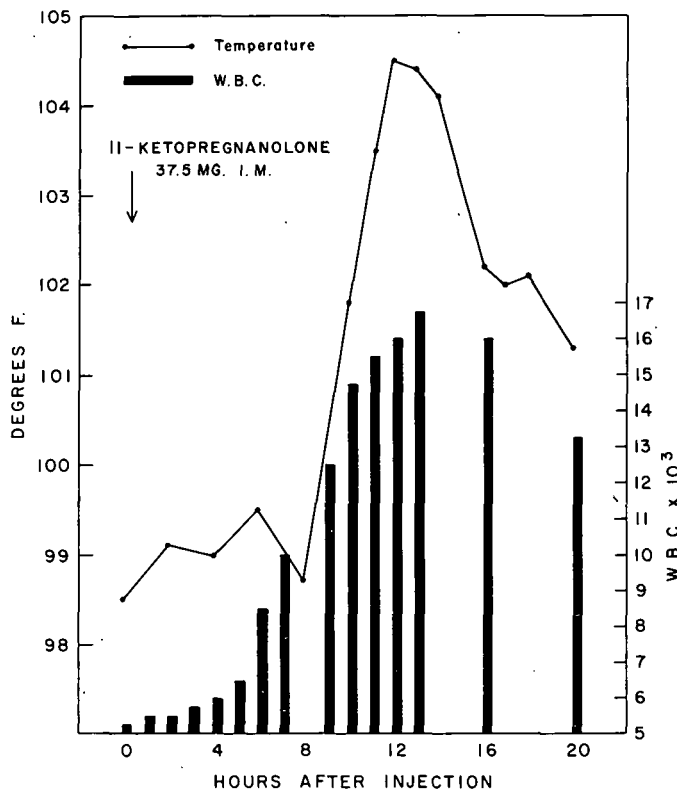
Figure 1. Typical polymorphonuclear leukocytosis accompanying the fever produced by pyrogenic steroids.

sex difference in response. Headache, malaise, fatigue, arthralgia and myalgia may accompany the febrile reaction and may be in part direct effects of the steroids themselves[18] as well as representing nonspecific responses to high fever. Local inflammatory reactions occur at the site of injection but both fever and local inflammation are transient.

STRUCTURAL BASIS OF THE PYROGENIC ACTION OF STEROIDS

Reductive transformation of the C 4-5 double bond is favored during in vivo metabolism of steroid hormones and this process leads to formation of a pair of isomeric compounds at carbon 5. In the 5a-H series the A:B ring junction is trans, or relatively planar, like that of the unaltered precursor hormone; in the 5β series, the A:B ring junction is cis, or highly angulated. Characteristically, steroids with powerful fever-producing activity in man belong to the latter category and may consist of compounds having 19, 21, or 24 carbon atoms. C19 and C21 steroid pyrogens represent metabolites derived from gonadal and adrenal hormones; C24 steroids are derived from degradation of cholesterol. The compounds shown in Figure 3 typify the structural
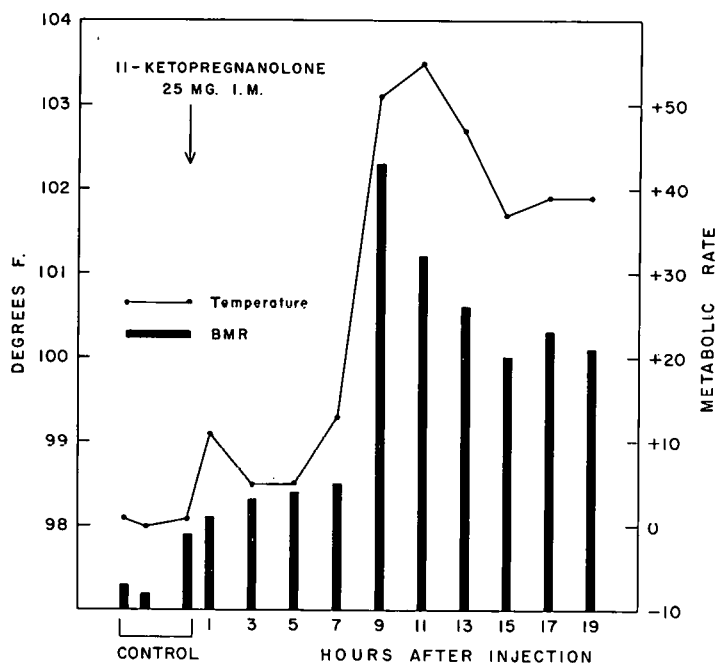
Figure 2. The calorogenic response to steroid-induced fever.

characteristics of such metabolites and represent the most potent fever-producing steroids known at present.

Other pyrogenic steroids have been described[19] which reflect the influence of structural alteration on the fever-producing capacity of the three basic compounds shown in Figure 3. Oxidation of the C3-hydroxyl group to a ketone yields compounds with reduced thermogenicity (etiocholanedione, pregnanedione); similar effects are noted with introduction of an 11β-OH group or an 11-ketone (11β-hydroxyetiocholanolone, 11-ketopregnanolone), although the C11-
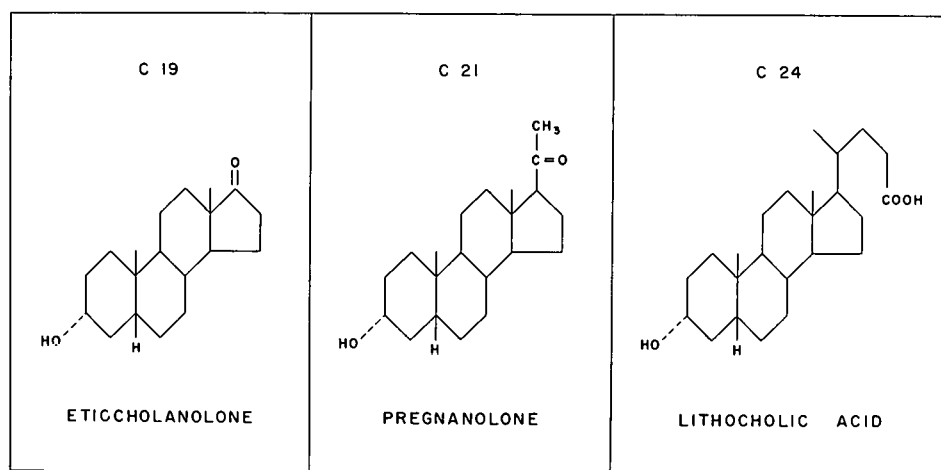
Figure 3. Structural characteristics of powerful fever-producing steroids of the 19, 21 and 24 carbon classes of metabolites.

ketone suppresses pyrogenicity to a considerably lesser degree than the 11β-hydroxyl. Reduction of the C20 ketone of pregnanolone to an alcohol reduces thermogenic capacity somewhat (pregnanediol); introduction of a C21 hydroxyl group (21-hydroxypregnanedione) yields a compound highly pyrogenic in a few subjects, but inert in most. Epimerization of the C3 hydroxyl or the C5 hydrogen atom abolishes (3β-etiocholanolone, isoandrosterone, 3β-allopregnanolone) or markedly suppresses (androsterone) thermogenic activity. Introduction of a 17α-OH group in the neutral steroids and of 7α-OH, 12α-OH, 7-keto or 12-keto groups in the bile acids also suppresses pyrogenicity. In the latter series of steroids, compounds with 6α or 7β hydroxyl groups have weak or irregular thermogenic action as well.[18]

INFLUENCE OF CONJUGATION ON THE PYROGENICITY OF STEROIDS

Steroid metabolites derived from in vivo transformation of cholesterol and of adrenal and gonadal hormones are found in plasma conjugated with a number of substances; the effect of these conjugation processes on fever production represents a special aspect of the influence of structure on the activity of these compounds. In the neutral series of steroid pyrogens (C19 and C21), chemical esterification (etiocholanolone acetate, pregnanolone acetate) or physiological conjugation (sulfate or glucosiduronate derivatives of etiocholanolone or 11-ketopregnanolone) results in complete loss of the pyrogenic activity which characterizes the free compounds. In the acidic steroid (bile acid) series of pyrogens (C24) these alterations do not abolish the biological activities which characterize the unconjugated steroid acids—for example, the 3-acetate and 24-methyl ether derivatives of lithocholic acid retain marked fever-producing action; similarly one of the physiological amino acid conjugates of this compound, glycolithocholic acid, provokes intense fever in man. Interestingly, another physiological conjugate of this compound, taurolithocholic acid, does not have pyrogenic action but retains the powerful inflammatory properties of the free bile acid.[20,18] Thus, conjugation does not uniformly suppress biological activity of steroid pyrogens although it may alter one or more expressions of this activity as demonstrated with taurolithocholic acid. Conjugation and "detoxification" therefore do not always have synonymous meaning and the latter can be assured only in certain circumstances by total removal, through excretion, of biologically active substances.

MECHANISM OF FEVER PRODUCTION

The mechanism by which steroid pyrogens act to produce fever in man is not known at present. Demonstration of a number of differences in the biological properties of these compounds and bacterial endotoxins,[21] however, suggests that the mode of action of these two classes of thermogenic agents may be different. Among the physiological differences which distinguish one class from the other, several are of special interest. The latent period between injection of steroids and onset of fever varies generally from four to eight hours; with bacterial endotoxins it is 20 to 90 minutes. Repeated injection of bacterial endotoxins rapidly leads to the development of "tolerance" to the biological actions of these substances; this phenomenon is not observed with steroid pyrogens. Bacterial pyrogens are irregularly effective when administered intramuscularly and consistently so (in appropriate doses) when injected intravenously; the converse is true with steroid pyrogens. Bacterial endotoxins have a wide species susceptibility. Steroid pyrogens are effective in producing fever only in man, a fact which emphasizes the potential bio-

logical significance of the well known species differences in secretion and metabolism of steroids and the need for caution in applying the results of studies in animals directly to human beings. Finally, the mode of action of bacterial endotoxins appears to involve leukocyte damage with release into the blood stream of a thermogenic protein—the "endogenous pyrogen" which acts proximally on thermoregulatory mechanisms and which is demonstrable by passive transfer of plasma from endotoxin-treated animals,[22-25] a comparable circulating pyrogen has not been detected in large volume (1000 ml) plasma transfers from patients with steroid fever. Demonstration of this transferable pyrogen in endotoxin fever in man, however, is difficult despite the likelihood that it is involved in the mechanism of such fever.[26] Thus, failure to demonstrate its presence in the plasma in steroid fever does not exclude this mode of action of steroids in thermogenesis. Nevertheless, for the present it is probably useful to accept the view that steroid pyrogens and bacterial endotoxins may well have different mechanisms of action.

The events which proceed during the prolonged latent period of steroid fever undoubtedly are essential to the mechanism of fever induced by steroids but they are at present obscure. In an attempt to identify these processes, the metabolic disposition of injected steroid pyrogen during this period has been examined in preliminary studies in this laboratory.[27] When tracer doses (nonpyrogenic amounts) of radioactively-labeled etiocholanolone are injected intramuscularly in man, there is rapid absorption of the steroid with peak blood levels appearing at approximately one hour; rapid urinary excretion follows and 50 per cent of injected radioactivity can be found in urine within four hours. Most of the remainder is recovered within 24 hours. When a radioactive tracer dose is administered in the same injection with a pyrogenic carrier dose of steroid, absorption is delayed and peak blood levels are reached at two to four hours. Urinary excretion is somewhat delayed also and significant radioactivity may persist in urine at 48 to 72 hours. When tracer and pyrogenic carrier doses are simultaneously injected at separate sites, absorption of tracer is rapid but excretion tends to be delayed. In all instances, labeled steroid can be found in cerebrospinal fluid in amounts proportional to plasma concentrations of the compound; in addition, peak plasma, cerebrospinal fluid and even urinary levels of injected steroid pyrogen have been attained well before the onset or peak of fever. Thus it is probable that high blood or cerebrospinal fluid levels of steroid pyrogen per se do not account for the thermogenic stimulus but that other factors occurring locally during delayed absorption or later during prolonged disposal of injected pyrogenic doses of steroid are involved in the mechanism of action of these compounds. In addition, despite the high recovery of unaltered steroid during metabolic experiments with these compounds, the possibility that the latent period may be attributed in part to the time required for transformation of injected steroid into a more active metabolite cannot be excluded.[28]

The production of local inflammatory lesions by steroid pyrogens is of interest in regard to the fever mechanism, apart from its intrinsic importance as a powerful biological activity of these steroids. Several observations suggest that nonspecific inflammation per se does not account for steroid-induced fever. Inflammatory reactions vary widely in time of onset and intensity of response following steroid pyrogen injection. Despite a general proportionality to dose, the local reaction in some patients may be slight with doses of steroid that produce intense fever; in addition, such reactions in other subjects may increase and persist long after the fever has subsided. Following intravenous injection of these steroids, phlebitis may develop concomitantly with or in the absence of fever; venous inflammation indeed has been reported as a frequent complication of intravenous administration of the nonpyrogenic steroid anesthetic, hydroxdione (Viadril: 21-hy-

droxypregnanedionsodium succinate).[29] Moreover, although fever production by these steroids appears limited to man, inflammation can be induced in certain animals; such reactions, however, are considerably less intense than those observed in human subjects. Finally, dissociation of inflammation from pyrogenicity has been shown with the steroid taurolithocholic acid—an observation consistent with others described above which suggest that these two steroid activities are independent even though generally associated. Nevertheless, these observations do not exclude the possibility that inflammation participates in some indirect way in the mechanism of fever produced by these compounds. Such participation could take the form of delaying absorption of injected steroid pyrogens, thus prolonging exposure of the thermoregulatory mechanisms to active steroids (or derivatives) or by specific interaction of steroid with tissue components or products of inflammation, resulting in formation of thermogenic products, the activity of which is ultimately terminated by disposal of steroid pyrogen through urinary excretion. The possibility that highly specific local processes of some type may be important in the mechanism of action of such steroids is supported by recent studies[27] indicating that relatively small amounts of the anti-inflammatory steroid cortisol markedly suppress fever when injected at the same local site as steroid pyrogen (Fig. 4); considerably larger doses of anti-inflammatory steroids injected at distant sites, however, often fail to affect the intensity of the thermogenic response. The nature of such processes or interactions between steroids of appropriate structure and local tissue components is at present unknown.
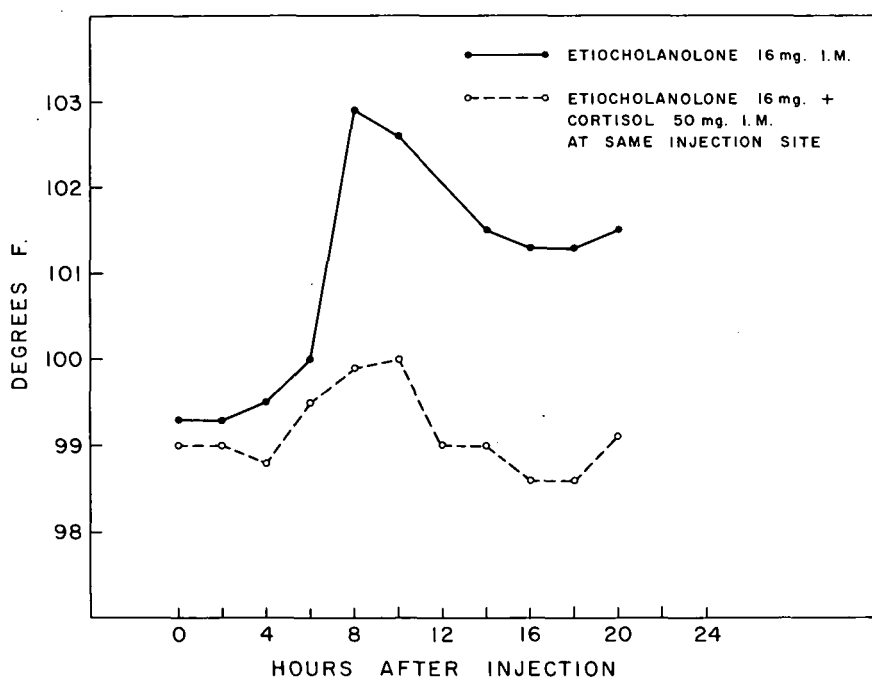

Figure 4. Suppression of the pyrogenic response to etiocholanolone by simultaneous local injection of the anti-inflammatory steroid, cortisol; larger amounts of cortisol injected at other sites have less fever-inhibitory action.

CLINICAL SIGNIFICANCE OF STEROID PYROGENS

The clinical significance of the fever-producing action of steroids derives from the endogenous origin of this new class of pyrogens; a natural interest has therefore developed in their possible participation in the mechanism of fever in certain clinical disorders in man.[16,30,31] This interest has been substantiated in the important studies of Bondy and associates on the relation of the pyrogen etiocholanolone to the pathogenesis of the febrile episodes of periodic fever.[12-14] Periodic fever is a descriptive term applied to an assortment of illnesses whose only recognized common feature is the recurrent development of episodes of high fever. In many patients these febrile episodes can be attributed to a variety of occult disorders such as infection, malignancy, etc.;[32] in others, conventional diagnostic efforts fail and no underlying disease can be identified.[33] In the latter group, Bondy et al.[12-14] have observed a number in whom high blood levels of unconjugated etiocholanolone have been noted during the febrile episodes of the disease; when the patients are afebrile, abnormal amounts of this steroid pyrogen are not detected in plasma. Patients with microbial fever do not demonstrate the steroidal abnormality nor is the presence of etiocholanolone in the plasma a non-specific consequence of fever per se. This biochemical abnormality therefore appears to be unique for this group of subjects and the recurrent episodes of fever are undoubtedly related in some way to the periodic appearance of unconjugated steroid pyrogen in the blood of these patients. The factors leading to this episodic occurrence of excessive production and defective conjugation of etiocholanolone are not known, nor for that matter is it clear that this represents the sole abnormality of steroid metabolism in such subjects since lack of appropriate methodology precludes more detailed examination of other possible defects in the biotransformation of steroids in these patients. The importance of these clinical studies, however, cannot be overemphasized since they may connote the existence of a number of febrile disorders in which abnormalities in production or metabolic disposition of endogenously produced steroid metabolites may play an important pathogenetic role.

It is important to recognize that the steroid metabolites described above have, in addition to their fever-producing capacities, potent hemolytic, cytotoxic and inflammatory properties as well[29] and these biological activities may express themselves clinically in the same manner as has been shown for the pyrogenic action of these compounds. In this regard, three clinical disorders may be of special interest. Recently Zieve[34,35] described in certain patients with liver disease a special type of hemolytic anemia, associated with hyperlipemia and low grade fever, which was different from the anemia observed in hypersplenism. The suggestion was made that abnormal lipids such as lysolecithin might be responsible for hemolysis in this disorder. It could be equally held that abnormal steroids (bile acids) circulating in the plasma might account for these findings since the serum bile acids in liver disease are altered in the direction of an increase in the actively hemolytic dihydroxy faction[36-38] and the major component of this fraction, chenodeoxycholic acid,[39] is metabolized to the potent hemolytic and pyrogenic compound, lithocholic acid.[40-42] Another disorder in which such steroids may play an etiologic role is acanthocytosis,[43-46] a genetically determined disease characterized by malformed erythrocytes, hemolysis, steatorrhea, hirsutism, menstrual irregularities and profound hypocholesterolemia (40 to 60 mg per cent). Apart from the clinical evidences of steroid abnormalities in this unusual disease (hirsutism, menstrual irregularities, hypocholesterolemia), the characteristic erythrocyte malformations have been reproduced in vitro with normal red blood cells exposed to ionic detergents;[47] the bile acid metabolites of cholesterol represent a major endogenous source of

such detergent-like substances and, in view of the derangements in cholesterol characteristic of acanthocytosis, examination of the qualitative and quantitative pattern of plasma bile acids may prove fruitful.

Finally, the cytotoxic and inflammatory properties of some steroids may be of importance in the etiology or progression of certain inflammatory intestinal disorders, and in cirrhosis of the liver. The intestinal tract is exposed continuously to large amounts of steroids derived from biliary excretion and enterohepatic circulation of these substances; among these steroids are such powerful cytotoxic compounds as lithocholic acid and its conjugates, and it is not difficult to conceive of circumstances in which these potent endogenous inflammatory agents act locally to impair the function, morphology and, indeed, viability of intestinal mucosa, with severe clinical consequences.[29] Less speculative grounds exist for seriously examining the role of cytotoxic bile acids such as lithocholic acid in the pathogenesis of cirrhosis of the liver in man since Holsti's important studies[48,49] have clearly demonstrated the cirrhogenic effect of this steroid, as well as several others, in experimental animals. Recent studies from this laboratory have confirmed these observations and further shown that other steroid pyrogens such as etiocholanolone have at least equally powerful cirrhosis-producing action.[27] These findings may indicate that the qualitative and quantitative alterations in blood levels of steroids (bile acids) observed in liver disease may not represent solely the consequences of the hepatic disorder itself, but may be involved directly in its progression or initiation.

SUMMARY

The in vivo metabolism of cholesterol and adrenocortical and gonadal hormones leads to the formation of a large number of steroid metabolites having potent fever-producing, hemolytic and cytotoxic properties. The pyrogenic activity of such metabolites has been implicated in the pathogenesis of fever in periodic disease and their hemolytic and cytotoxic properties may participate also in the mechanism of other febrile and inflammatory disorders. Examination of the possible relation of these endogenous steroid metabolites to such diseases represents a potentially fruitful area of clinical investigation.

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EFFECTS OF STEROID SEX HORMONES ON IMMUNOLOGICAL PHENOMENA[*]

By

A. Kappas, H. E. H. Jones,[†] and I. M. Roitt[†]

Gonadal hormones markedly inhibit the growth, maturation and metabolism of lymphoid tissues[1]—effects which might be reflected in impaired immunological reactivity in animals treated with these hormones. We have examined this possibility in several experimental contexts.

Continued administration of œstrone to female guinea pigs injected with homologous thyroglobulin in complete Freund's adjuvant greatly diminished the intensity of induration of the delayed skin reactions to both tuberculin and thyroglobulin (Table 1). The erythematous reactions to thyroglobulin were not depressed, and this may be related to the lack of effect of œstrone treatment on circulating antibody titres in these animals. The severity of the thyroiditis lesions[2] was unaffected in one group of twelve guinea pigs examined and was slightly reduced in a second group of seven.

Table 1

EFFECT OF ŒSTRONE ON DELAYED SKIN REACTIONS[‡] IN GUINEA PIGS
INJECTED WITH HOMOLOGOUS THYROGLOBULIN (Tg) IN
COMPLETE FREUND ADJUVANT

Treatment	Test antigen	No. of animals	Erythema			Induration		
			-	+	++	-	+	++
Control	P.P.D.	18	0	3	15	5	9	4
Œstrone (0.5-1.0 mg daily)	P.P.D.	19	8	6	5	17	2	0
Control	Tg	18	8	8	2	6	9	3
Œstrone (0.5-1.0 mg daily)	Tg	19	8	7	4	14	5	0

[‡]Skin reactions were read 24 h after intradermal challenge.

Comparable experiments in female rats involving the production of auto-allergic thyroiditis[3] gave more variable results: œstrone markedly reduced the severity of thyroid lesions in three experiments but was without effect in two others (Table 2). We do not, at present, understand the reasons for the inconsistency of the steroid effect, although the wide scatter in the severity of thyroiditis induced by auto-immunization in each group of rats might be a contributory factor. The tuberculin responses were more consistently affected and, despite the difficulty of grading the skin reaction in this species, the results were indicative of a similar trend to that seen in the guinea pig. In these animals, moreover, there was clear-cut suppression of adjuvant-induced ar-

Table 2

EFFECT OF SEX STEROIDS ON THE PRODUCTION OF AUTO-ALLERGIC THYROIDITIS IN RATS

Treatment		Mean thyroiditis score*		Per cent inhibition	Significance of difference (=P)
		Control	Treated		
Œstrone (0.5-1.0 mg daily)	Exp. 1	1.5 ± 0.6 (6)	0.5 ± 0.2 (6)	67	0.1
	Exp. 2	1.5 ± 0.4 (6)	0.2 ± 0.2 (6)	87	0.02
	Exp. 3	2.5 ± 0.5 (4)	2.3 ± 0.3 (12)	None	-
	Exp. 4	1.0 ± 0.5 (5)	1.5 ± 0.6 (4)	None	-
	Exp. 5	1.0 ± 0.4 (7)	0.1 ± 0.1 (7)	90	0.1
Testosterone propionate (0.5-1.5 mg daily)	Exp. 1	1.2 ± 0.4 (6)	0.6 ± 0.3 (12)	50	0.2 ⎫ < 0.05
	Exp. 2	1.2 ± 0.3 (6)	0.2 ± 0.2 (5)	85	< 0.05 ⎭

*The degree of thyroiditis was arbitrarily scored as follows: no lesion, 0; mild focal, 1; moderate diffuse, 2; severe with goitre, 3. The arithmetic mean score ± S.E. is given. The figures in parentheses refer to the number of rats in the group. Each experimental group had separate controls to allow for variations in the adjuvant mixture, diet, etc.

thritis;[4] whereas inflammatory joint changes were evoked in eight out of twenty-eight control rats injected with the adjuvant mixture alone, no arthritis was seen in a similar group of thirty-five animals given œstrone daily. In a larger series of one hundred and forty-four rats, less intensive œstrogen administration diminished the incidence of adjuvant-induced arthritis by 50 per cent.[5] With regard to other immunologically dependent phenomena, administration of œstrone did not influence the course of rejection of skin homografts or the incidence of experimental allergic encephalitis in single experiments involving groups of six rats.

An immuno-suppressive effect was also observed in rats with testosterone which clearly inhibited the development of auto-allergic thyroiditis in males (Table 2), and reduced the incidence and severity of adjuvant arthritis in both males and females.[5] It remains to be seen whether these effects are related to the hormonal nature of the steroids or to a property of the molecular structure independent of their classical hormonal actions. The sex hormone effects on experimental adjuvant arthritis may have clinical implications and this possibility is being investigated.

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THE EFFECT OF ERYTHROPOIETIN UPON UTILIZATION OF GLUCOSAMINE BY MARROW CELLS IN CULTURE*

By

P. P. Dukes, F. Takaku,[†] and E. Goldwasser

It is now widely accepted that the hormone erythropoietin is an important regulatory factor in the process of red blood cell formation. Several recent reports on the action of erythropoietin on erythropoietic tissues in vitro have been for the main part concerned with the incorporation of precursors into heme or hemoglobin or with the gross uptake of radioiron into cells (Erslev,[1] Powsner et al.,[2] Korst et al.,[3] Krantz et al.[4]).

We shall report here, briefly, some observations on another, as yet incompletely defined, biochemical process which is affected by erythropoietin. Marrow cells in short term culture incorporate glucosamine-1-C^{14} into an insoluble product, which we have tentatively assumed to represent material derived from cell membranes and stroma. The biosynthesis of this fraction from glucosamine is significantly stimulated by the addition of erythropoietin to the culture medium.

METHODS AND MATERIALS

Marrow cells from the femora and tibiae of 200 g male Sprague-Dawley rats were suspended in the medium at a final concentration of about 10^7 cells per ml. The medium consisted of equal parts of NCTC 199 and unfiltered calf serum which had been heated at 56° for 30 minutes. It also contained 40 units of penicillin G per ml, 25 μg of streptomycin per ml, 0.02 μmoles per ml of glucosamine-1-C^{14} HCl (5.1 μC per μmole); and erythropoietin where indicated. Each incubation mixture had a final volume of 2.0 ml. The cultures were gassed with a stream of 5 per cent CO_2, 95 per cent air for one minute before the tubes were closed and incubated at 37°. After incubation the cells were washed free of medium with cold saline, then washed successively with 5 per cent trichloracetic acid, alcohol, and alcohol:ether 1:1. The residue was dissolved in performic acid; aliquots were plated on weighed stainless steel planchets as infinitely thin samples and counted in a Tracerlab thin window "Omniguard" counter. All results were expressed as cpm per mg of dry residue. The sheep plasma erythropoietin (step III lot 137) was obtained from the U. S. Public Health Service Study Section on Hematology and assayed by the fasted rat method (Fried et al.[5]). The units of activity are those defined previously (White et al.[6]).

RESULTS AND DISCUSSION

Marrow cell cultures incorporated glucosamine into the insoluble fraction continuously during the first 48 hours of incubation. In the presence of 3.5 units per ml of erythropoietin, this incorporation was significantly increased (Fig. 1). Both the control and stimulated curves
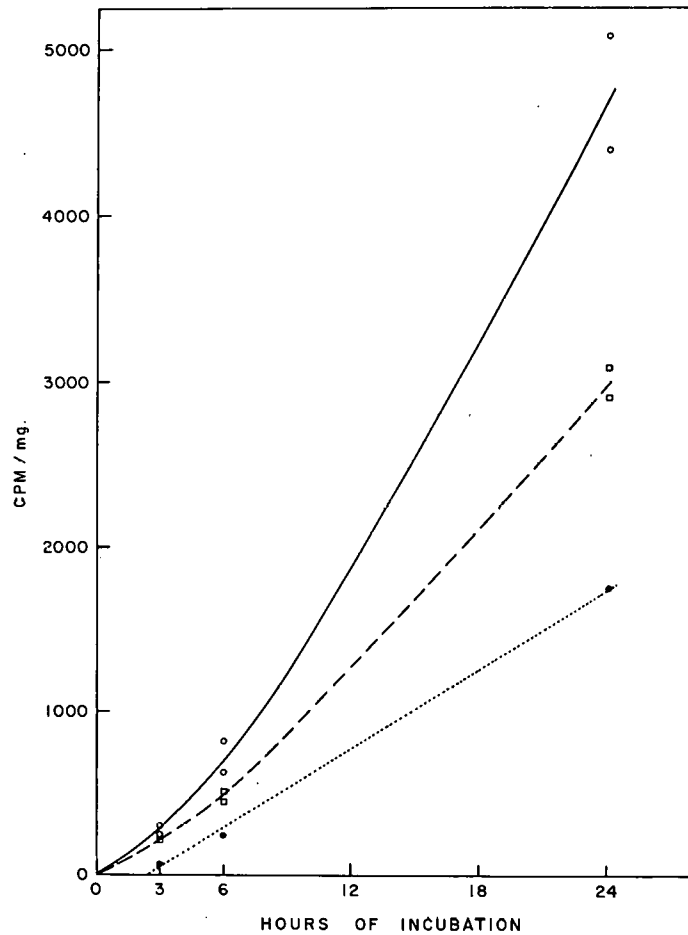
Figure 1. Time course of erythropoietin effect
on glucosamine-1-C^{14} uptake into marrow cells
in culture: o——o with erythropoietin; □- - - -□
without erythropoietin; ●······● difference curve.

are curvilinear with an upward concavity and appear to represent initially an exponential course
of incorporation for the control and an increment added to the control curve which represents
the stimulated condition. The difference between the two curves is linear with the time and can
be extrapolated to zero difference at 2.5 hours. These data suggest that the effect of erythropoi-
etin on glucosamine incorporation is indirect, and that the direct effects are exerted in the first
2.5 hours of incubation of the cultures under these conditions. In each of three other experiments
with varying times of incubation, the difference curve extrapolated to zero at about 2.5 hours.

The effect of amount of erythropoietin upon glucosamine incorporation was studied at 24
hours, with the results shown in Figure 2. A plot of the response, in cpm per mg, versus the
logarithm of the amount of erythropoietin has the sigmoid shape expected for hormone action.
This type of curve may represent the increased probability, with increasing amount, of success-
ful encounters between hormone molecules and sensitive cells.

With some preparations of erythropoietin the dose-response curve shows a decline at lev-
els of about 1 unit per ml, suggesting that an inhibitory substance is present in these impure
fractions. These observations obviously limit the use of glucosamine incorporation as an assay
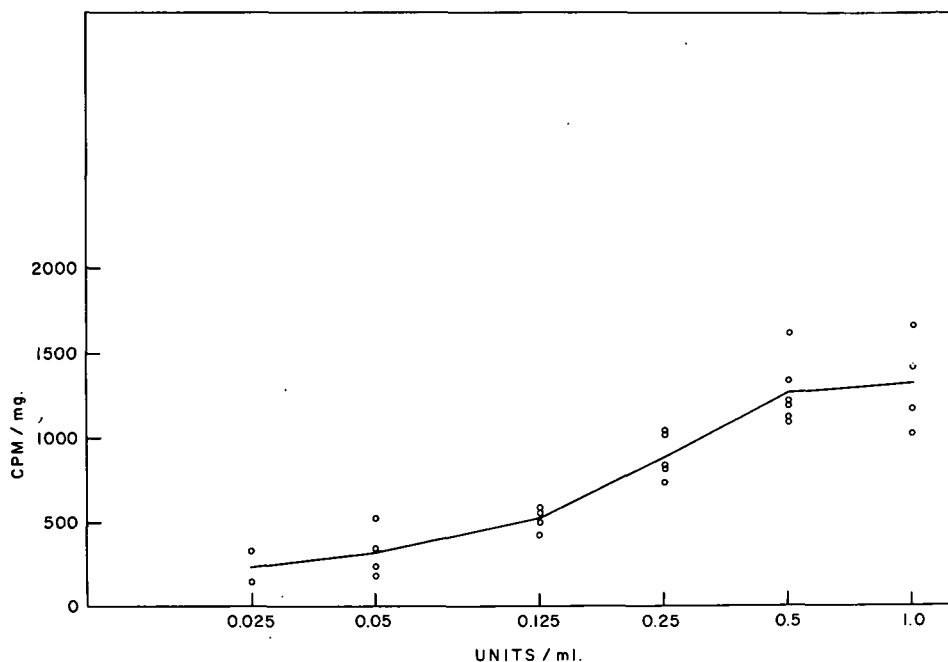
Figure 2. Relation of glucosamine uptake to erythropoietin concentration.

method for erythropoietin, except in a highly purified state.

The identity of the radioactive material synthesized by these cells from glucosamine is not yet established, but we have evidence showing that about 30 per cent of the insoluble radioactivity is in the form of N-acetyl neuraminic acid and/or N-glycolyl neuraminic acid.

More extensive data on the specificity of erythropoietin action on marrow utilization of glucosamine will be published in the near future.

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EFFECT OF RADIATION ON INTERMEDIARY METABOLISM
OF THE RAT[*]

By

J. H. Rust,[†] G. V. LeRoy, J. L. Spratt,[‡] Gar Bo Ho, and L. J. Roth[†]

"Enzyme inactivation can . . . be studied in the intact animal by administering a metabolite marked with ^{14}C and studying the amount of labeled CO_2 exhaled previous to and after exposure. If irradiation reduces the amount of labeled CO_2 exhaled, this does not prove an inactivation of the enzymes involved; viz, the effect of exposure may be due to other reasons than enzyme inactivation and insofar as the latter took place it may be difficult to ascertain which of the enzymes involved in the catabolic process was influenced. If, however, irradiation does not affect the amount of exhaled CO_2 we can conclude that the enzyme system involved in the catabolic process was not influenced by exposure—or more correctly, that a sufficient fraction of the enzyme involved remained intact and can thus perform its task at a normal rate."

G. Hevesy, 1951

While studying the effects of protracted, fractionated gamma radiation on burros, Rust and his colleagues observed increased blood pyruvate, and histologic changes in adrenal and thyroid glands which suggested that irradiation altered the metabolic state of these animals.[1] Further studies were therefore carried out to investigate energy metabolism. In 1955, Lane, Wilding, Rust, Trum and Schoolar[2] reported that the respiratory quotient (RQ) of burros was lowered during protracted, fractionated total-body gamma radiation delivered at rates of 25 and 200 r per day. The reduction of RQ to less than 0.5 was of such magnitude that only a profound physiologic disturbance could account for the changes measured. Although oxygen consumption was reduced somewhat, reduction of the respiratory excretion of carbon dioxide was primarily responsible for the altered RQ.

Since these changes were opposite to what was expected, the present studies were carried out, using rats, to examine intermediary metabolism in greater detail than was convenient using burros. At that time (1954) there was relatively little information on the metabolic effects of irradiation on intact animals. It was reported that in rodents there was little change in basal oxygen consumption;[3,4] that the urinary excretion of urea, ammonia and uric acid was increased;[5-8] that there was a moderate increase in the fasting blood glucose concentration;[9] that there was a transient increase in the liver glycogen of fasted animals that was maximal on the second day post-irradiation;[10,13] and that there was a moderate decrease in the amount of exhaled CO_2.[14] The literature contained many reports of the effects of ionizing radiation on tissue slices, tissue homogenates, isolated cells and separated enzyme systems; but it was difficult to determine what role such changes might play in the metabolism of intact animals.

Our studies were concerned with the nature of the disturbance of intermediary metabolism responsible for the decreased output of CO_2—and thus of the lower RQ—following total-body irradiation. The two reports which follow are complementary, differing chiefly with respect to the methods used in the studies.

PART I[*]

Previous reports from Rust's laboratory described methods for studying the incorporation of C^{14} from labeled bicarbonate, glucose and alanine into the liver glycogen, urea, and expired air of normal—i.e., unirradiated—rats;[15] and the catabolism of urea in irradiated rats.[16]

METHODS

Female Sprague-Dawley rats weighing 70 - 90 g were used for all experiments. Food, but not water, was withheld from the time of irradiation until the completion of the tracer metabolic study. Non-irradiated controls were starved for a comparable period of time. For each experiment the total number of animals was divided into groups of 4 or 6 using a table of random numbers. They were assigned for irradiation or as controls by a throw of a die. Animals were placed in lucite boxes and exposed to the gamma rays of the Co^{60} teletherapy unit of the Argonne Cancer Research Hospital. The distance from the source was 80 cm, and the dose rate was 20 r/min measured in air using calibrated Victoreen ionization chambers.

First experiment. Thirty rats were exposed to 1000 r, and 30 controls were sham-irradiated in groups of 6 animals each. Forty-eight hours later 6 irradiated and 6 control rats were placed in individual Roth metabolism units[17] for 18 hours to measure the output of CO_2 in expired air. The air supply to the sealed glass chambers passed through Ascarite absorbers to remove ambient CO_2. The outflowing air was directed through a pair of gas-absorbing towers, in series, that contained 5N NaOH. The amount of CO_2 produced by each animal was determined manometrically with the Van Slyke apparatus using an aliquot of the alkali from the two absorbers.

Second experiment. Eighteen rats were exposed to 1000 r, and 18 were sham-irradiated in groups of 6 each. Forty-eight hours later 6 treated and 6 control animals were given intraperitoneal injections of one of the labeled substrates: $NaHC^{14}O_3$, uniformly labeled glucose-C^{14}, or alanine-2-C^{14}. The characteristics of the tracers and the dosages used are given in Table 1. Immediately after injection each rat was placed in an individual Roth metabolism unit. Urine and feces were collected separately, and CO_2 in expired air was trapped in the gas-absorbing towers as described above. Single rats from both treated and control groups were killed at 1, 2, 3, 4, 5 and 7 hours after injection.

Liver glycogen was extracted immediately by the hot KOH-methanol method, and assayed by the anthrone method of Seifter, et al.[18] The specific activity (SA)—dpm per mg of carbon—of glycogen was determined after wet oxidation using the Van Slyke-Folch method.[19] The CO_2 generated was collected in a liquid N_2 trap on the vacuum line. After measurement in the Van Slyke manometric apparatus, the gas was transferred to a Borokowski[20] ionization chamber and the radioactivity measured with a vibrating reed electrometer.

SA of expired CO_2 was determined similarly after measurement of the amount of CO_2 re-

Table 1

CHARACTERISTICS OF THE LABELED SUBSTRATES

	Tracer	Dose, per 100g of body weight	
		Mg	μc
Part I	$NaHC^{14}O_3$	2.19	1.27
	Glucose-C^{14}*	2.85	0.15
	Alanine-2-C^{14}	4.66	0.18
Part II	$NaHC^{14}O_3$	0.005, 0.014	0.19, 0.50
	Na Formate-C^{14}	0.024, 0.048	1.00, 2.00
	Na Acctate-1-C^{14}	0.046	1.09
	Na Acetate-2-C^{14}	0.018	2.57
	Glucose-C^{14}*	0.14	2.00
	Glucose-1-C^{14}	0.34	2.23
	Glucose-6-C^{14}	0.19	0.98
	Fructose-C^{14}	0.068	3.23
	Ribose-1-C^{14}	0.14	2.07

*Uniformly-labeled with C^{14}.

Notes: (1) Carbohydrate tracers were checked chromatographically, and kept frozen until used. For Part II, tracers were assayed twice, in duplicate using the Tri-Carb liquid scintillation spectrometer. Radioassays were calibrated with standard solutions of toluene-C^{14} or cholesterol-4-C^{14}.

(2) For intraperitoneal injection, the volume was 1.0 ml per 100g of body weight; for intravenous injection the volume was 0.33 ml per 100g.

leased from the alkali of the absorbing towers by H_3PO_4. The amount of SA of urea in urine was determined by the Conway microdiffusion method.[21] Carbon dioxide released by the action of urease was absorbed by 0.5 N NaOH in the central wall. An aliquot of this was analyzed in the Van Slyke apparatus and the gas then transferred to the ionization chamber.

Third experiment. Eight rats were exposed to 1000 r, and 8 controls were sham-irradiated in groups of 4 animals each. At 24 and 48 hours one irradiated group and one control group were studied to determine the size and rate of turnover of the CO_2 pool. After intraperitoneal injection of $NaHC^{14}O_3$ the rats were placed in individual Roth metabolism cages. The alkali in the absorbing towers was changed at frequent intervals, and SA of CO_2 was determined on successive aliquots. At the end of the test period all animals were bled from the heart, and the CO_2 content of whole blood was measured in the Van Slyke manometric apparatus. Pool size was calculated from the y-intercept of the extrapolated die away curve of SA of CO_2; and turnover time was estimated from the slope of the curve.

Radioassay procedures were calibrated using standard solutions of C^{14}-labeled sodium bi-

carbonate; and stable carbon assays were calibrated using benzoic acid, both obtained from the National Bureau of Standards.

RESULTS

First experiment. Preliminary studies demonstrated considerable individual variation among irradiated and fasted control rats in the amount of CO_2 exhaled per hour when the time of collection was of short duration. However, the results indicated that the lowest value for CO_2 output occurred during the 2nd and 3rd days post-irradiation. For the 30 control rats the mean output of CO_2 was 1.65 ± 0.21 ml/hour per 100 g. The mean value for 30 irradiated rats 48 hours after 1000 r was 1.26 ± 0.26 ml/hour per 100 g. This represents an average reduction of the output CO_2 to 76 per cent of the control value.

Second experiment.

1. The per cent of dose of bicarbonate-C^{14} recovered from expired air, and from urine urea, was not significantly different in the irradiated and the control animals (see Table 2). Each

Table 2

PER CENT OF DOSE OF TRACER-C^{14} RECOVERED AFTER INTRAPERITONEAL INJECTION, 2ND EXPERIMENT, PART I

Duration, hours	Per cent of dose of C^{14} in:					
	Expired air		Urea		Glycogen	
	Control	Irrad.	Control	Irrad.	Control	Irrad.
(Tracer: NaHC^{14}O$_3$)						
1	73.9	61.4	0.34	0.07	0.006	0.032
2	74.7	64.2	0.61	0.77	0.007	0.20
3	83.5	78.3	0.73	1.29	0.007	0.76
4	84.4	80.9	0.85	0.40	0.015	0.72
5	86.5	81.2	0.89	0.54	0.007	0.66
7	84.2	83.4	0.93	1.30	0.006	0.44
Average: 3-7 hrs.	84.7	81.0	0.85	0.88	0.009	0.65
(Tracer: Glucose-C^{14})						
1	5.3	4.3	0.32	0.15	1.1	1.8
2	23.1	17.9	1.31	0.26	0.9	1.1
3	31.7	27.8	1.97	0.42	0.9	2.6
4	33.7	24.6	1.20	0.69	0.4	4.0
5	35.8	43.0	1.77	-	0.4	2.6
7	32.4	37.6	0.57	0.75	-	3.2
Average: 3-7 hrs.	33.4	33.3	1.38	0.62	0.57	3.10
(Tracer: Alanine-2-C^{14})						
1	4.7	2.7	0.7	0.2	1.5	0.7
2	17.6	15.6	3.2	1.0	2.9	4.1
3	20.9	26.4	2.5	0.9	1.4	4.8
4	-	23.2	-	1.1	1.3	3.1
5	25.9	21.4	1.8	1.7	0.7	0.6
7	28.2	20.4	2.2	0.9	0.5	3.0
Average: 3-7 hrs.	25.0	22.9	2.2	1.2	1.0	2.9

entry in this table represents one rat and the period during which exhaled CO_2 and urine were collected ranges from 1 to 7 hours. Inspection of the data suggests that the per cent of dose recovered (and for SA, in Table 3) for individual rats was approximately the same after the 2nd hour. Accordingly the values for the 3rd, 4th, 5th, and 7th hours are averaged. The lack of influence of irradiation on the per cent of dose of bicarbonate recovered in expired air is confirmed by results reported in Part II, Tables 6 and 7.

Table 3

"RATIO OF CONTRIBUTION"; SA PRODUCT/SA PRECURSOR
AFTER INTRAPERITONEAL INJECTION,
2ND EXPERIMENT, PART I

Duration, hours	SA_{urea}/SA_{CO_2}		$SA_{glycogen}/SA_{CO_2}$	
	Control	Irrad.	Control	Irrad.
(Tracer: $NaHC^{14}O_3$)				
1	0.3	0.07	0.001	0.007
2	0.6	0.80	0.001	0.005
3	0.7	1.30	0.001	0.009
4	0.8	0.40	0.001	0.013
5	0.9	0.50	0.003	0.014
7	0.9	1.30	0.004	0.015
Average: 3-7 hrs.	0.83	0.88	0.002	0.013
(Tracer: $Glucose-C^{14}$)				
1	2.2	0.4	0.18	0.42
2	3.4	0.5	0.11	0.34
3	4.7	0.7	0.14	0.59
4	3.9	1.4	0.16	0.90
5	4.5	0.8	0.18	0.31
7	3.0	0.9	0.26	0.59
Average: 3-7 hrs.	4.02	0.95	0.19	0.60
(Tracer: $Alanine-2-C^{14}$)				
1	2.4	0.7	0.11	0.25
2	4.0	1.5	0.04	0.45
3	3.7	1.3	0.06	0.97
4	-	6.0	-	-
5	4.9	5.0	0.07	0.23
7	5.7	5.0	0.08	-
Average: 3-7 hrs.	4.77	4.33	0.07	0.60

2. The per cent of dose of bicarbonate-C^{14} incorporated—or fixed—in liver glycogen in the control rats ranged from 0.006 to 0.015 (average 0.009) for the period 3 - 7 hours after injection of the tracer. In irradiated rats, 48 hours after 1000 r, the per cent of dose incorporated in liver glycogen ranged from 0.032 at 1 hour to 0.76 at 3 hours after injection; the average value (Note: emphasis is desirable for fixation of CO_2) for the period 3 - 7 hours after injection increased more than 70 fold (see Table 3). When the specific activity (SA) of CO_2 is compared to the SA of glycogen, the ratio of contribution (SAglycogen:SA_{CO_2}) is 6 times greater in the ir-

radiated then in the control animals. The difference between the SA ratios (X6) and the ratios of fraction of dose (X72) indicates that glycogen is being stored by the liver as well as being synthesized at a greater rate in the irradiated animals.

3. When uniformly labeled glucose-C^{14} was injected intraperitoneally, the fraction of $C^{14}O_2$ (called the apparent rate of oxidation, ARO) during periods ranging from 1 to 7 hours was the same in irradiated and control animals (see Table 2). The fraction of glucose carbon incorporated into urea was reduced by a factor of 2 post-irradiation, while the fraction incorporated into liver glycogen was increased 5-fold, and in one rat as much as 4 per cent of the injected dose of glucose was recovered from liver glycogen. Table 3 demonstrates that the ratio, $\underline{\text{SA glycogen: SA}}_{CO_2}$ is increased 3-fold in the irradiated rats, while the ratio, $SA_{urea}:SACO_2$ is decreased 4-fold. It appears, therefore, that CO_2 derived from the oxidation of glucose is utilized preferentially for fixation in glycogen at the expense of fixation in urea.

4. Alanine-2-C^{14} was used as a convenient equivalent to pyruvate. When its apparent rate of oxidation was examined (Table 2) there was no difference between irradiated and control rats although 5 of the 6 individual values for irradiated rats were lower than the controls. As in the case of glucose, the fraction of alanine carbon recovered as urea was reduced by a factor of 2 post-irradiation. The per cent of alanine carbon incorporated into liver glycogen was approximately 3 times greater in the irradiated animals. In the fasted controls, the per cent of alanine carbon incorporated into liver glycogen in the process of gluconeogenesis ranged from 0.5 to 2.9 (see Table 3). The ratio, $SA_{urea}:SACO_2$ was the same in irradiated and control animals. but the ratio, $\underline{SAglycogen:SACO_2}$ was increased 8-fold (Table 3).

Third experiment. Efforts to evaluate the CO_2 pool were not entirely satisfactory for technical reasons, and results should be considered provisional. Nevertheless, the irradiated and control animals were treated in the same fashion and the results shown in Table 4 can be compared. It appears that the CO_2 pool post-irradiation is approximately 25 per cent smaller than

Table 4

THE CARBON DIOXIDE POOL, AVERAGE VALUES

	N	Pool size mg C/100 g	Turnover time, hours	Whole blood CO_2, mM/liter
Controls fasted for 48 hours	4	4.55	5.4	24.9
48 hours after 1000 r, fasted	4	3.40	5.0	30.2

in the controls, although the turnover time (which appears to be rather long) is the same. The content of CO_2 in whole blood was about 20 per cent greater 48 hours post-irradiation. It seems proper to conclude that radiation has not affected the movement of CO_2 from tissues to plasma to alveolar air to a significant extent.

Our findings in irradiated rats may be summarized as follows:

(a) The amount of CO_2 in expired air is decreased.

(b) Fixation of CO_2 in liver glycogen is increased.

(c) The recovery of CO_2 in expired air from bicarbonate, and the fixation of CO_2 from bi-

carbonate in urea is not affected.

(d) Incorporation of carbon from glucose and alanine into liver glycogen is increased.

(e) Incorporation of carbon from glucose and alanine into urea is diminished.

(f) Glycogenesis and gluconeogenesis are increased.

PART II[*]

With methods developed in the Argonne Cancer Research Hospital[22] for continuous monitoring of the activity of $C^{14}O_2$ in expired air it was possible to examine systematically one aspect of intermediary metabolism of intact rats following irradiation—namely, the rate of conversion of specific substrates to CO_2.

When an intermediary metabolite labeled with C^{14} is administered and the activity of $C^{14}O_2$ in expired air as a function of time is determined using the continuous radiation monitor, we obtain information on the apparent rate of oxidation of that particular substrate. The term apparent rate of oxidation (ARO) is preferred because, although the true rate of oxidation per unit of time is not actually measured, it is assumed to be proportional to the rate of elimination of $C^{14}O_2$ in expired air. Factors other than the true rate of intracellular oxidation—such as variations in the size and turnover of metabolic pools, or changes in biosynthetic reations—may be affected by irradiation and influence the fraction of dose of C^{14} recovered per unit of time, thus modifying the ARO. While any alteration in the size of the miscible pool of a particular substrate cannot be measured directly by this method, it is often possible to make reasonable deductions about a particular pool on the basis of the behavior of several substrates that may contribute to it.

The plot of $C^{14}O_2$ in expired air versus time yields curves that are complex since the carbon tracer of the substrate necessarily traverses several metabolic pools. One of these is the CO_2 pool which is the final common pathway for all CO_2 produced by intracellular oxidation. Within a group of healthy animals, the configuration of the time-course of the elimination of $C^{14}O_2$—and the fraction of tracer carbon recovered per unit of time—appears to be unique and quite consistent for each of the labeled intermediates used in this study. If ionizing radiations have a significant effect on the activity of any of the enzymes involved in oxidative metabolism, we would expect some change in the metabolic pathways utilized by such substrates as bicarbonate, formate, acetate, ribose, fructose and glucose. The changes may be manifested by alterations of the ARO as evidenced by (1) the activity versus time curve, or (2) variation in the fraction of dose of C^{14} recovered in some specified time—such as 1 hour.

METHODS

Prior to irradiation young female Sprague-Dawley rats weighing approximately 75 g (77 ± 11 g) were fed the standard rat ration ad libitum. Food, but not water, was withheld from the time of irradiation until the completion of the tracer metabolic study. In series F and G food was withheld for 24 hours before irradiation and until the completion of the tracer study. The non-irradiated controls were similarly treated.

Irradiation was by a General Electric unit operated at 250 KVP with a filter of 0.5 mm of copper and 1.0 mm of aluminum. The half-value layer of the beam was equivalent to 1.45 mm

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[*]These studies were carried out in Argonne Cancer Research Hospital.

of copper. The dose rate, measured in air, was 53 to 55 r/min using calibrated Victoreen ionization chambers. During exposure the rats were restrained in individual plastic cylinders rotating slowly at a distance of 75 cm from the target.

At times ranging from immediately to 72 hours after irradiation an animal was injected with a solution of one of the tracers listed in Table 1. The control rats received an equivalent dose by the same route. Promptly after injection the rat was placed in a sealed glass container supplied with a constant flow of 225 ml/min of dried air from a tank. The outflow from the animal container passed through a drying tube packed with anhydrous $CuSO_4$, and then to the $4-\pi$ Geiger-Mueller counter (Model 4P5, Instrument and Developments Products Co., Chicago) which continuously monitored the radioactivity of expired air. The output of the counter actuated a ratemeter and a graphic recorder, while a scaler connected in parallel accumulated the counts throughout the experiment. Figure 1 is a block diagram of the apparatus. The counting-gas was helium saturated with ethanol at $0°$ C, flowing at a constant rate of 177 ml/min through

CONTINUOUS $C^{14}O_2$ MONITOR
(ARGONNE CANCER RESEARCH HOSPITAL)
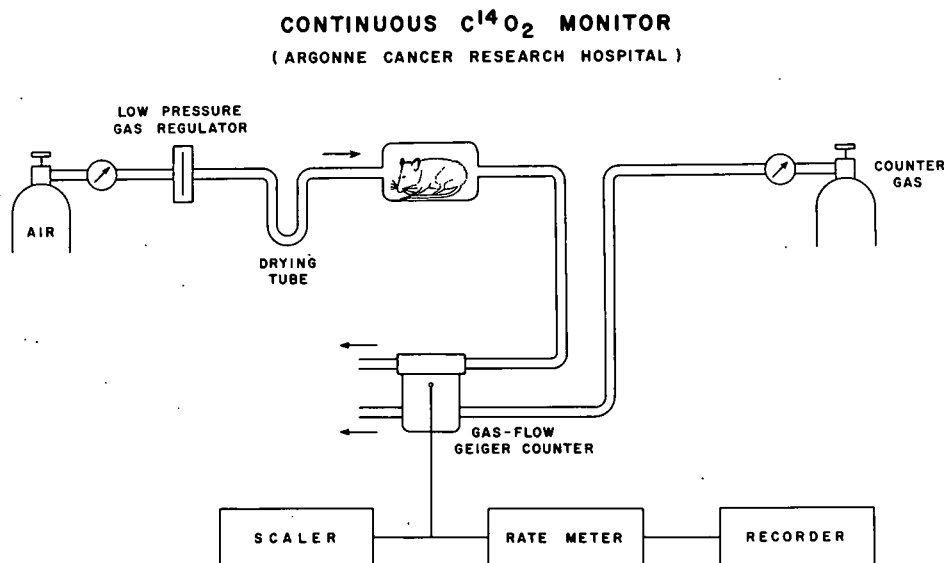
Figure 1. Block diagram of apparatus.

the detector. The apparatus was calibrated daily using compressed air containing a known amount of $C^{14}O_2$. The efficiency of the two monitors used was 33.8 ± 1.13 per cent and 29.8 ± 1.11 per cent, respectively. Following the tracer experiment the animals of series A and D were returned to their cages and allowed to feed ad libitum. They were observed daily until they died and all survivors were killed after 30 days. The rats of the other series were killed immediately after the tracer study.

Animals were assigned to irradiation or control groups in a random fashion. Early in the study the measurement of C^{14} activity in expired air was continued for 2 to 3 hours after injection. Analysis of the data demonstrated no important difference between the values for 1 hour and those for later intervals, therefore all results reported apply only to the first hour following administration of the tracer. All results are reported as per cent of dose of C^{14} recovered from expired air during the first hour.

The 30-day mortality rate for the rats of series A and D was as follows: 200 r - 21.3 per cent, 400 r - 43.9 per cent, and 800 r - 96.0 per cent. The number of animals in the 3 dose groups was 94, 107, and 176, respectively. The estimated LD_{50}-30 days, for 250 KVP x-rays, was 450 r.

Series A. In this experiment 3 rats were used for each tracer-time-dose group. The data are given in Table 5. Analysis of variance was performed using the per cent of dose of bicarbonate recovered at 1 and 2 hours. This revealed that starvation increased the recovery of

Table 5

PER CENT OF DOSE RECOVERED 1 HOUR AFTER INTRAPERITONEAL ADMINISTRATION, SERIES A, PART II

Tracer	Time of test, hours*	Dose of X-rays, r			
		0	200	400	800
Bicarbonate	0	51.2[†]	46.7	59.3	47.0
	6	51.8	45.3	49.6	36.4
	12	51.3	51.4	53.2	52.5
	24	56.5[‡]	72.6	76.9	51.6
Formate	0	31.7	30.5	25.1	31.2
	6	24.1	28.8	29.8	25.3
	12	21.7	30.1	27.2	25.8
	24	21.6[‡]	21.7	23.3	21.8
Acetate-1	0	46.8	37.9	54.5	40.7
	6	45.9	49.8	43.6	38.0
	12	40.3	40.2	39.5	44.1
	24	45.9	41.7	44.4	46.6
Acetate-2	0	30.6	24.2	19.9	23.6
	6	22.7	25.0	27.8	26.5
	12	33.9	24.4	30.2	28.2
	24	31.6	20.9	28.8	27.1

*After irradiation or sham-exposure.

[†]Each entry is the average of the results on 3 rats.

[‡]The effect of 24 hours' starvation is significant at the 95 per cent confidence level.

$C^{14}O_2$ from bicarbonate-C^{14}, and decreased the ARO of formate-C^{14} at the 95 per cent level of confidence. Starvation did not alter the ARO of acetate significantly. Irradiation had no statistically significant effect on the metabolism of any of these substrates during the first 24 hours after exposure. The lack of significant differences between interactions among groups, and the internal estimates of variance within groups suggests that there was no marked bias in the study.

Series B. Because some workers[1,23] have noted that irradiation may alter intraperitoneal absorption of certain metabolites, the experiments of series A were repeated using intravenous injections of the tracers. The time of study after exposure was extended to 48 hours since no effect due to irradiation had been observed at 24 hours. Table 6 shows that the recovery of $C^{14}O_2$ from bicarbonate is not altered by irradiation: the ARO of carboxyl-labeled acetate is signifi-

Table 6

PER CENT OF DOSE RECOVERED 1 HOUR AFTER INTRAVENOUS
ADMINISTRATION, SERIES B AND C, PART II

Tracer	Time of test, hours	Dose of X-rays, r								
		0			400			800		
		N	Mean ± SD*		N	Mean ± SD		N	Mean ± SD	
Bicarbonate	24	6	75.1	8.6	6	70.7	11.9	6	71.4	4.9
	48	12	60.8	5.4	6	61.3	4.5	12	61.5	5.6
Acetate-1	24	10	47.2	3.3	10	46.5	2.7	9	47.9	6.8
	48	9	46.3	3.5	10	46.0	4.2	9	39.8[†]	2.7
Acetate-2	24	6	30.0	2.6	7	30.6	2.1	7	23.9[‡]	1.5
	48	6	29.5	2.1	12	33.3	5.8	12	23.0[†]	1.0
Formate	24	10	22.0	3.2	10	27.8[‡]	3.0	11	27.8[‡]	1.0
	48	10	22.0	3.3	12	24.2	3.6	10	27.1[‡]	1.4
	72	10	26.3	4.9	10	28.1	4.3	14	28.3	4.0

[*]Standard deviation $= \sqrt{\dfrac{\Sigma(x)^2 - \dfrac{(\Sigma x)^2}{N}}{N - 1}}$.

[†]P = < 0.001 for difference between 800 r and 0 r groups.

[‡]P = < 0.005 for difference between irradiated and 0 r groups.

cantly reduced at 48 hours after 800 r (P = < 0.001); and the ARO of methyl-labeled acetate is also decreased at 24 hours (P = < 0.005) and 48 hours (P = < 0.001) after 800 r, but not after 400 r.

Series C. When formate-C^{14} was given intravenously the per cent of dose recovered during 1 hour was significantly increased at 24 and 48 hours. Accordingly, additional experiments were performed at 72 hours. The mean per cent of dose recovered was increased, but the difference from the controls was not statistically significant. The data are given in Table 6.

Series D. When specifically-labeled glucose was given intraperitoneally there appeared to be a trend in the direction of decreased ARO at successive intervals of time between 6 and 24 hours irradiation. At 24 hours the P-value for the difference between irradiated and control animals was 0.01 for glucose-1-C^{14}, but was only 0.10 > P > 0.20 for glucose-6-C^{14}. The ratio, $C^{14}O_2$ from C_6:$C^{14}O_2$ from C_1 (i.e., C_6/C_1) was not affected significantly. The results are given in Table 7.

Series E. When glucose, fructose and ribose were given intravenously and their ARO measured at 4, 24 and 48 hours after irradiation there was a significant progressive decrease in the fraction of dose of C^{14} recovered in the 1-hour experiment. The results are shown in Table 11. In the case of glucose the C_6/C_1 ratio was not altered significantly at any time, as shown also in Table 8.

Series F and G. These experiments were performed to investigate a discrepancy between our findings with fructose and some data reported by Rust[1] who observed a marked reduction

Table 7

PER CENT OF DOSE RECOVERED 1 HOUR AFTER INTRAPERITONEAL
ADMINISTRATION, SERIES D, PART II

| Tracer | Time of test, hours | Dose of X-rays, r | | | | | |
| | | 0 | | | 800 | | |
		N	Mean ± SD		N	Mean ± SD	
Glucose-1	0	6	12.0	1.0	6	11.0	1.6
	6	6	12.2	1.2	7	12.0	2.0
	12	7	11.4	2.4	7	10.3	1.7
	24	7	11.9	1.6	6	9.4*	1.3
Glucose-6	0	6	8.6	1.1	6	9.9	1.3
	6	6	9.7	1.6	6	10.7	2.0
	12	7	9.3	1.1	6	8.6	1.2
	24	6	8.5	2.3	7	7.1	1.0
Ratio: C_6/C_1	0	.72			.88		
	6	.79			.90		
	12	.81			.83		
	24	.72			.76		

*$P = < 0.01$.

in the ARO of fructose-C^{14} following irradiation. His rats were starved for 24 hours before irradiation with 1000 r of Co^{60}-gamma rays, and the metabolic study was performed 48 hours later, after intraperitoneal injection of the fructose. The rats in series F and G, Part II, were also starved for 24 hours before irradiation and tested 48 hours later. Series F were injected intraperitoneally and series G intravenously, with uniformly labeled glucose or fructose. The results are given in Table 9.

The additional 24 hours of starvation had a significant effect on the ARO of fructose, reducing the per cent of dose recovered from 12.7 to 7.0, in the unirradiated controls when the dose was given intravenously. After irradiation the per cent of dose recovered depended on the route of administration: 4.8 after intraperitoneal, and 6.9 after intravenous. The lower value for ARO after intraperitoneal injection is in agreement with Rust's findings. In the case of glucose the additional 24 hours of starvation had no significant effect on the ARO of either the irradiated or the control animals.

To summarize: following total-body irradiation with doses in the lethal range (800 r) the apparent rate of oxidation of acetate, glucose, fructose and ribose was reduced to a significant extent at 48 hours. The greatest reduction occurred in the case of glucose-6-C^{14}. At the same time interval the ARO of formate was significantly increased. No difference was observed when bicarbonate-C^{14} was the tracer. Expressing all the results—when the tracers were given intravenously—as fractions of the values obtained in the non-irradiated controls gives the data shown in Table 10.

DISCUSSION

It is appropriate to combine the discussion of the results of the experiments described in Parts I and II since both were performed to seek an explanation for the decreased output of CO_2

Table 8

PER CENT OF DOSE RECOVERED 1 HOUR AFTER INTRAVENOUS ADMINISTRATION, SERIES E, PART II

Tracer	Time of test, hours	Dose of X-rays, r								
		0			400			800		
		N	Mean	± SD	N	Mean	± SD	N	Mean	± SD
Glucose[*]	4	8	20.0	5.1				7	20.3	3.5
	24	7	13.1	1.0	8	14.4	1.8	7	11.6	1.6
	48	16	12.8	2.0	6	11.2	1.1	15	$\underline{9.7}$[†]	$\underline{1.3}$
Glucose-1	4	8	15.9	1.8				7	17.2	2.1
	24	6	9.8	0.6	7	11.0	1.2	6	10.9	1.2
	48	6	11.6	1.1	6	9.7	1.2	6	$\underline{8.5}$[†]	$\underline{0.8}$
Glucose-6	4	8	11.6	1.2				7	13.0	1.9
	24	8	7.9	2.8	10	8.0	2.1	6	7.9	1.2
	48	14	8.8	2.8	6	7.6	0.6	13	$\underline{5.0}$[†]	$\underline{0.9}$
Fructose[*]	4	6	13.6	4.0				6	12.7	3.7
	24	7	12.8	1.2	9	13.7	1.7	7	12.4	1.1
	48	8	12.7	2.0	8	11.4	1.8	9	$\underline{9.4}$[†]	$\underline{1.3}$
Ribose-1	4	8	17.1	2.1				8	15.9	2.4
	24	8	11.2	1.8	8	10.5	2.1	9	10.5	1.5
	48	7	9.8	1.5	7	8.6	0.8	8	$\underline{7.5}$[†]	$\underline{1.0}$
		Ratio	±	SD[‡]	Ratio	±	SD	Ratio	±	SD
C_6/C_1	4	0.73		0.12				0.75		0.15
	24	0.81		0.29	0.73		0.21	0.72		0.12
	48	0.76		0.25	0.78		0.12	0.59		0.12

[*]Uniformly labeled.

[†]$P = < 0.005$ for difference between 800 r and 0 r.

[‡]SD of ratios $= R\sqrt{\dfrac{S_1^2}{\overline{X}_1^2} + \dfrac{S_2^2}{\overline{X}_2^2}}$, when $R = C_6/C_1$ ratio; $S_{1,2}$ for glucose-6, and glucose-1, respectively; and $\overline{X}_{1,2}$ = Mean per cent for glucose-6, and glucose-1, respectively.

in the expired air of irradiated animals. The finding—in burros— of RQ values well below those ordinarily expected suggested that radiation influences intermediary metabolism to a much greater extent than had been appreciated. The major emphasis in the discussion will be focused on results observed at 48 hours after 800 r of x-rays, or 1000 r of Co^{60} gamma rays. These doses are well in excess of the MLD for the young female rat, and are about the LD_{95} for the Sprague-Dawley strain. The effects of lower doses were inconclusive with the number of animals studied. Tracer studies were seldom performed later than 48 hours post-irradiation (except for series

Table 9

INFLUENCE OF ROUTE OF ADMINISTRATION ON PER CENT OF DOSE
RECOVERED IN 1 HOUR; SERIES F AND G, PART II

Tracer	Route of injection	Dose of X-ray, r					
		0			800		
		N	Mean	± SD	N	Mean	± SD
Glucose[*]	Intraperitoneal	6	9.6	1.5	6	8.7	0.7
	Intravenous	9	12.4	2.5	10	9.1[†]	2.4
Fructose[*]	Intraperitoneal	9	7.2	1.5	8	4.8[†]	0.8
	Intravenous	7	7.0	1.3	7	6.9[‡]	1.1

[*]Uniformly labeled material.

[†]$P = < 0.005$ for difference between 800 r and 0 r groups.

[‡]$P = < 0.005$ for difference between intraperitoneal and intravenous groups.

Table 10

SUMMARY OF DATA ON RECOVERY OF $C^{14}O_2$ IN 1 HOUR, EXPRESSED AS THE

FRACTION: $\dfrac{\text{Mean per cent after 800 r}}{\text{Mean per cent after 0 r}}$ PART II

Tracer	Time of test after irradiation			
	4 Hours	24 Hours	48 Hours	P[*]
Glucose[†]	1.02	0.88	0.76	0.005
Glucose-1	1.08	1.11	0.73	0.005
Glucose-6	1.12	1.00	0.57	0.005
Fructose[†]	0.93	0.87	0.74	0.005
Ribose-1	0.93	0.94	0.77	0.005
Acetate-1		1.02	0.86	0.005
Acetate-2		0.80	0.78	0.005
Formate[‡]		1.27	1.23	0.005
Bicarbonate		0.95	1.01	.40 < .30

[*]Student's t test for contrast between controls and 800-r group at 48 hours.

[†]Indicates uniformly labeled material.

[‡]At 72 hours after irradiation the fraction was 1.08.

C of Part II) because by that time rats exposed to an LD_{95} show severe injury to the hematopoietic system and the gastrointestinal tract, bacterial invasion of tissues is beginning, and they are approaching the moribund state. Furthermore, at 48 hours the concentration of glycogen in the liver is usually maximal,[10] as is the concentration of cholesterol and fatty acids.[24-26]

Table 11

CHANGES IN LIVER AND MUSCLE GLYCOGEN 48 HOURS AFTER
1000 r OF $Co^{60}\gamma$-RAYS[*]

		N	Fasted controls	N	Irradiated
A.	Liver weight, as per cent of body weight	18	3.80 ± 0.50[†]	30	5.12 ± 0.28[‡]
B.	Liver glycogen content, as mg per 100 g of body weight	33	42 ± 14	29	87 ± 31[‡]
C.	Concentration of glycogen in liver, per cent[**]		1.1		1.7
D.	Concentration of glycogen in muscle, per cent	24	0.167 ± 0.018	29	0.173 ± 0.015

[*] Data from University of Chicago Thesis of S. S. Tom, 1956.

[†] Mean ± standard deviation.

[‡] P = > 0.001.

[**] Calculated from measurements of separate groups of rats: B ÷ A, above.

1. CO_2 in Expired Air

The first experiment, Part I, confirmed that irradiated rats behaved in the same fashion as mice[14] and burros:[2] that is, the amount of CO_2 in expired air was significantly decreased. Basal O_2 consumption was not measured but we assume that our rats were similar to those studied by others[3,4] who have reported little or no change during the first 2 or 3 days post-irradiation. The usual value reported for basal O_2 consumption is 2.0 ml/hour/100 g of rat. If our controls were normal, their RQ should have been about 0.82 (1.65/2.00 = 0.82), a reasonable value after 48 hours fasting. If the O_2 consumption of the irradiated rats was also normal, their RQ must have been less than 0.71, and may have been as low as 0.62 (1.26/2.00 = 0.62). Even if the basal O_2 consumption 48 hours after 1000 r had been reduced by as much as 10 per cent, the RQ of the irradiated rats would still have been less than 0.71. According to classical concepts of physiological chemistry, the RQ reflects the composition of the mixture of substrates oxidized to yield energy, and an RQ of 0.71 suggests that oxidation of fat is the "sole" source of CO_2 in expired air.

We interpret the results of the first and third experiments (Part I)—which demonstrated no significant influence of radiation on the CO_2 pool—to mean that the reduced output of CO_2 post-irradiation cannot be attributed to a disturbance in the movement of CO_2 from tissue cells to plasma to alveolar air. This conclusion is supported by the results reported in Part II, where the fraction of dose of bicarbonate recovered in expired air was the same in irradiated and control rats (Tables 5, 6, 7 and 10).

2. Oxidation of Substrates

The apparent rate of oxidation (ARO) was investigated using C^{14}-labeled glucose, fructose, ribose, alanine, acetate and formate. The experiments reported in Part I used a time-consuming system involving the collection of CO_2 in gas absorbing towers, and the subsequent radioassay of CO_2 released from the alkali absorbent. The method is inherently insensitive to small

variations in the time-course of the oxidation of the substrates. The results obtained with glucose-C^{14} and alanine-2-C^{14} (Table 2) suggested little or no difference between irradiated and control rats when the period of measurement ranged from 3 to 7 hours after injection of the tracer.

The experiments in Part II used a highly efficient monitor for the continuous measurement of $C^{14}O_2$ in expired air. Preliminary studies indicated that the first few hours—in most instances, the first hour—after injection of the tracer provided the most useful information on the ARO. When the measurements were continued over several hours, differences that were evident at the outset became progressively smaller. The substrates used trace the principal pathways of intermediary metabolism of carbohydrates, fats, and 1-carbon compounds. The method is sufficiently sensitive to distinguish the behavior of carbon-1 of glucose (which is oxidized promptly via the pentose phosphate pathway), from that of carbon-6 (which is oxidized more slowly because of the longer pathways traversed by carbon atoms of the trioses) (see Tables 7 and 8). Similarly, the method is sensitive enough to display the expected difference between the ARO of the carboxyl carbon of acetate and that of the methyl carbon (see Tables 5 and 6). Accordingly, if radiation selectively affected a key enzyme of the pentose phosphate pathway (for example) the result should be a significant change in the ARO of glucose-1-C^{14} as well as a change in the C_6/C_1 ratio if glucose-6-C^{14} was also tested. The metabolic activity examined by this procedure is the composite result of oxidative processes occurring throughout the intact animal.

Experimental conditions were designed to facilitate collection of suitable data for statistical analysis. In series A, 3 rats were used in each dose-time-substrate group to maximize the likelihood of demonstrating bias—if it existed—in the design of the experiment. The analysis of variance suggested that there was no marked bias. In all other series the rats in each group ranged from 6 to 16, a number large enough to allow the use of Student's t test to evaluate the significance of the difference between means of irradiated and control animals.

A. Carbohydrate. The effect of irradiation on the ARO of glucose was studied carefully because of our initial conviction that one or more of the pathways of glucose metabolism might be disturbed to a significant extent. When tracer glucose was injected intraperitoneally the ARO was not significantly affected by radiation, although a trend is evident in Table 7. At 48 hours, using uniformly-labeled glucose-C^{14} intraperitoneally, the ratio of ARO in irradiated and controls was 0.99 (Table 2) by the method described in Part I, and 0.90 by the method described in Part II (Table 9). When the glucose-C^{14} was injected intravenously in two separate series (Tables 8 and 9)—the ARO of the irradiated rats was 73 and 75 per cent of the fasted controls respectively. Clearly there is an advantage to intravenous injection of the tracer material. When specifically labeled glucoses—glucose-1-C^{14}, and glucose-6-C^{14}—were given intravenously, the ratio, irradiated: control was 0.73 and 0.57 respectively. All these values for ARO in irradiated animals are significantly different from the control (P = < .005) when glucose was given intravenously, but they do not differ significantly among themselves. Thus, although the ARO of glucose-6-C^{14} appears reduced to a greater extent (0.57) than the ARO of glucose-1-C^{14} (0.73), the ratio of C_6/C_1 in the irradiated rats is not significantly different from the controls. Ribose-1-C^{14} and uniformly labeled fructose behaved in a similar fashion, as shown in Table 8. Therefore, we conclude that the ARO of the carbohydrate tracers used was reduced uniformly post-irradiation to about 75 per cent of the values in fasted controls. However, even if the reduced

apparent rate of oxidation represented a comparable reduction of the true rate of oxidation of carbohydrates, such a moderate decrease was not sufficient to account for values for RQ less than 0.71. Furthermore, we believe that there is a better explanation for the decreased ARO of the carbohydrate tracers than impairment of the ability to oxidize glucose. The conclusion that oxidation of glucose was not impaired to an important extent following irradiation is consistent with the results reported by Weber and Cantero.[12] In addition to this they found changes in the behavior of hepatic enzymes involved in the metabolism of glucose-6-phosphate typical of increased glycogenesis and gluconeogenesis.

B. Acetate. The ARO of methyl-labeled and carboxyl-labeled acetate was decreased to 78 and 86 per cent, respectively, of the control values of 48 hours after 800 r (Table 6). Since acetate—or more properly, acetyl-CoA—is the product of fat which is oxidized, we had anticipated that the ARO of tracer acetate would be increased and that increased utilization of fat as a source of energy might account for some of the reduced output of CO_2 and the decrease of the RQ. This was a reasonable expectation since others have reported evidence of increased mobilization of fat* and increased biosynthesis of liver cholesterol and liver fatty acids from acetate post-irradiation.[24,25] Since the ARO of acetate was decreased, it is possible that the pool of acetyl-CoA was enriched by acetate from depot fat to a greater extent than oxidation was increased, so that the tracer study gave the results observed. However, we have not performed any experiments to explore such an explanation of the reduced ARO of tracer acetate.

3. Fixation of CO_2 and Transfer Rates

The second experiment, Part I, was designed to provide information on the SA (dpm per mg C) of three of the products—CO_2 in expired air, urine urea, and liver glycogen—derived during intermediary metabolism from the precursors: bicarbonate, glucose and alanine. The method was suggested, in part, by some work of Kleiber and his associates[26-28] on the role of fixation of CO_2 during the synthesis of milk by the dairy cow. Briefly, if one injects a precursor (such as $NaHC^{14}O_3$) which has several possible products: viz. plasma CO_2, alveolar CO_2, urea, etc.; and if equilibration occurs during the period of observation, one can use the "ratio of contribution," viz.: SA product:SA precursor to draw some conclusions about the rate of transfer of carbon, or the extent to which a particular precursor is the source of carbon for a particular product. The ratio, SA alveolar CO_2:SA plasma CO_2 is assumed to be unity, for obvious reasons. The fraction of urea carbon derived from plasma CO_2 (see Table 3) is given by the ratio, SA urea:SA CO_2 which increased from 0.3 at 1 hour to 0.9 at 5 - 7 hours. From these data one can conclude that plasma CO_2 may have supplied as much as 90 per cent of the carbon used for urea synthesis. However, when glucose-C^{14} was the source of carbon, SA urea exceeded SA CO_2 by a factor of 4 in the control rats, indicating that carbon can be transferred from glucose to urea by some other pathway than via plasma CO_2. After irradiation this pathway practically ceased to operate, so that the ratio, SA urea:SA CO_2 was only 0.95, or about what would be expected if bicarbonate was the only source of the carbon. At present, we have no information about this pathway, which was so drastically modified by radiation.

When the source of carbon was alanine-2-C^{14} the ratio, SA urea:SA CO_2 was not affected by radiation. This ratio in both irradiated and controls was in excess of 4. Therefore, the reac-

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*Personal communication from E. P. Cronkite, present address Brookhaven National Laboratory.

tion for the transfer of carbon from alanine to urea was not sensitive to radiation, while that for glucose carbon was seriously compromised. This conclusion is further supported by the data on the fractions of dose of the three substrates recovered from urea. As shown in Table 2, the fraction from bicarbonate was not changed, that from alanine was reduced somewhat, and that from glucose reduced by a factor of 2. Again we have no information about the enzyme systems responsible for these events.

The data for liver glycogen were striking. When bicarbonate-C^{14} was the tracer, the average fraction of dose fixed in glycogen was 72 times greater in the irradiated animals (see Table 2). With glucose-C^{14}, the fraction incorporated into liver glycogen increased 5-fold, and with alanine, there was a 3-fold increase. Since it is known that liver glycogen increases post-irradiation, the results with glucose and alanine were not unexpected since this was what should occur when glycogenesis increased. The enhanced fixation of CO_2 in glycogen, however, was quite unexpected, since the reactions involved: carbohydrate → pyruvate → glycogen; and alanine → pyruvate do not involve fixation of CO_2. Gluconeogenesis from proteins via aspartic acid and glutamic acid does not involve fixation of CO_2, and the reaction oxaloacetate → pyruvate yields CO_2. The fixation of CO_2 in glycogen was further documented by the transfer rate between plasma CO_2 and glycogen. The ratio, SA glycogen:SA CO_2 was increased 6 times post-irradiation. Furthermore CO_2 derived from the oxidation of glucose and alanine participated in the fixation reaction as shown by the ratios, SA glycogen:SA CO_2 which were increased by factors of 3.2 and 8.6 respectively post-irradiation.

All these data confirm that glycogenesis is increased, and lead to the conclusion that gluconeogenesis must be occurring by a mechanism that requires the fixation of CO_2. Such a pathway is probably the reaction: Acetyl-Co A + CO_2 → Pyruvate.

That liver glycogen is increased post-irradiation has been reported by several workers. Lourau and Lartigue[13] attributed the increase to enhanced glycogenesis; Weber and Cantero[12] suggested that it was due in part to gluconeogenesis even though they found no impairment of glucose metabolism; McKee and Brin[29] considered that gluconeogenesis was the principal reaction responsible for increased liver glycogen because the increase failed to occur in hypophysectomized or adrenalectomized animals; Tom[10] found that the concentration of liver glycogen 48 hours after 1000 r of Co^{60} gamma rays was about twice that of fasted controls, whereas muscle glycogen was only slightly increased. He also found that liver glycogen was not increased following irradiation of adrenalectomized rats. Some of his data are of particular interest (Table 11) since he used the strain of rat and the procedures described in Part I, and worked in the same laboratory.

Our experiments provide no clues to the manner in which radiation (1) increases glycogenesis by the liver, (2) increases gluconeogenesis from amino acids, and (3) increases gluconeogenesis from acetyl-Co A. It is likely that the latter reaction is the most important of the three since it appears to account adequately for the decreased output of CO_2 in expired air and the reduced RQ. The changes in ARO of the carbohydrate and acetate tracers are probably best explained by the demands of glycogenesis and by augmentation of the metabolic pools of acetyl-Co A and pyruvate. In this connection, it is of interest that elevated levels of blood pyruvate have been reported following irradiation in several species.* We have no information about the path-

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*Personal communication from B. F. Trum—present address Harvard Medical School, Boston, Massachusetts.

way for transferring carbon 'directly' from glucose to urea which appears to be seriously impaired post-irradiation.

Formate is probably not involved in reactions leading to pyruvate $\rightleftharpoons$ acetyl-Co A except as another source of CO_2. The increased ARO of formate suggests that irradiation does not depress all oxidative processes indiscriminately; and the increased recovery of $C^{14}O_2$ from labeled formate supports the conclusion that the CO_2 pool is not significantly affected by radiation—a conclusion indicated by the fact that the recovery of labeled bicarbonate was the same in irradiated and control animals (see Tables 2, 5 and 6). Finally, the increased ARO of formate may represent an increased availability of that substrate for oxidation when a competing metabolic process—nucleic acid synthesis—is drastically curtailed by irradiation.

CONCLUSION

Viewed as a whole, our findings do not confirm the supposition that some of the harmful or lethal effects of radiation are caused by injury to intracellular enzymes responsible for energy production by the oxidation of carbohydrates, fats and proteins. Our findings are consistent with the reports of other workers who have described other disturbances of intermediary metabolism, namely: (1) decreased CO_2 in expired air, (2) increased biosynthesis of cholesterol and fatty acids in the liver, (3) increased glycogenesis, and (4) increased gluconeogenesis.

It seems that radiation has a greater influence on anabolic processes—which it appears to enhance—than on catabolic, or oxidative, reactions. We conclude, therefore, that ionizing radiation has an effect on the intermediary metabolism of the rat, the importance of which cannot be evaluated on the basis of our findings.

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STUDIES ON THE INTERACTION BETWEEN BOVINE SERUM ALBUMIN AND NATURAL AND SYNTHETIC POLYRIBONUCLEOTIDES
I. THE PREVENTION OF THE THERMAL COAGULATION OF BOVINE SERUM ALBUMIN BY NATURAL AND SYNTHETIC POLYRIBONUCLEOTIDES[*]

By

S. Yachnin[†]

More than a decade ago it was shown that the thermal coagulation of bovine serum albumin (BSA) could be prevented by desoxyribonucleic acid (DNA).[1] Limited complex formation has been demonstrated between DNA and native BSA under restricted conditions of ionic strength and pH by both light scattering[2] and electrophoresis.[3] Subay and Doty[4] demonstrated that the protection against the heat coagulation of BSA afforded by DNA depends upon the formation of a large complex between the two macromolecules. Since this complex could be dissociated by 8 M urea, they concluded that complex formation was not due to simple electrostatic interaction, but rather was dependent upon hydrogen bonding.[4]

Synthetic polynucleotides by themselves are immunologically inert,[5] whereas BSA is a relatively potent antigen. In the hope that a complex between these substances stabilized by hydrogen bonds might elicit the formation of antibodies specific for the polynucleotide moiety, a systematic study of their interaction was undertaken. The results described in this paper demonstrate that under appropriate conditions, polyribonucleotides can inhibit the heat coagulation of BSA.

METHODS AND MATERIALS

Crystalline BSA was purchased from Armour Pharmaceutical Corporation, North Chicago, Illinois. Polyadenylic acid (Poly A), polyuridylic acid (Poly U), polyinosinic acid (Poly I) and polycytidylic acid (Poly C) were synthesized in this laboratory utilizing polynucleotide phosphorylase isolated from Micrococcus lysodeikticus.[6] Highly polymerized calf thymus DNA was a product of the Sigma Chemical Company, St. Louis, Missouri. RNA was prepared from rabbit and rat liver by the method of Laskov et al.[7] Ribonuclease (5 x crystallized) was purchased from the Nutritional Biochemicals Corporation, Cleveland, Ohio.

All buffer solutions were prepared from reagent grade salts. When preparing BSA-polynucleotide mixtures, BSA was generally used in a final concentration of from 1.5 to 3 mg/ml; polynucleotide concentration varied from approximately 0.5 to 1 mg/ml. In all instances appropriate blanks and controls were employed. Freshly prepared solutions were used in all experiments.

Heat denaturation was accomplished by immersing the solutions contained in stoppered glass tubes in a boiling water bath (100° C) for 10 min and allowing them to cool at room temperature. Turbidity was either assessed by inspection, or was measured by determining ab-

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sorbancy at 700 mμ. Protein determinations were done by a modified Folin method.[8] Polynucleotide concentrations were measured by the orcinol method[9] or by ultraviolet absorption. All pH measurements were made with a Beckman Zeromatic pH meter. A Zeiss PMQ II spectrophotometer and cuvettes with a 1-cm light path were used throughout.

RESULTS

The behavior of BSA in solution. When heated in 0.145 M NaCl, 0.015 M phosphate buffer, pH 7.4, solutions of BSA remain clear. When heated in more dilute citrate and phosphate buffer solutions turbidity begins to appear at approximately pH 6.0, and below this pH coagulation is virtually complete with the formation of a coarse contracted coagulum (making nephelometric measurements impossible) and the absence of residual protein in the supernatant as measured by the Folin technique. Thus any protective effect of polynucleotide on visible heat coagulation of BSA must be sought below pH 6.0.

The behavior of native BSA-polynucleotide mixtures in solution. Goldwasser noted that when BSA and DNA were mixed, precipitation occurred on the acid side of the isoelectric point of the protein and that mixtures of the two substances were turbid up to pH 5.2.[3] Similar phenomena occur when polyribonucleotides and BSA are mixed in 0.0015 M citrate buffer. If the ionic strength of the buffer was raised (0.005 M, 0.05 M), the pH at which turbidity first appeared was lowered in all instances, but gross precipitation always occurred as the isoelectric point of the protein (pH 4.5) was approached. When analyzed, the insoluble material always contained both polynucleotide and BSA, and could be redissolved by the addition of salt.

The effect of heat upon mixtures of BSA-polynucleotide. Varying amounts of polynucleotide were mixed with BSA at pH 5.5; the solutions were heated, observed for turbidity, and in certain instances when heat coagulation occurred, the sediment was centrifuged and the clear supernatant analyzed for BSA and polynucleotide content. The results (Table 1) indicate that both natural and synthetic polyribonucleotide can completely inhibit the heat coagulation of up to 3 times their weight of BSA.

The next series of experiments was designed to study the effects of pH and ionic strength upon the interaction between BSA and polynucleotide. The results are shown graphically in Figure 1. It can be seen that all the polynucleotides tested were capable of protection against BSA heat coagulation in 0.0015 M citrate buffer. Protection was also afforded in 0.005 M citrate buffer, but raising the buffer concentration to 0.05 M abolished this effect. It will be noted that the precipitates formed when the mixtures were heated contained both BSA and polynucleotide (Table 1), and could not be redissolved by raising the salt concentration or pH.

The effect of Mg^{++}. The effect of Mg^{++} upon the protective action of RNA against BSA heat coagulation can be examined in Table 2. 10^{-3} M Mg^{++} completely abolished this protective effect, while lower concentrations of Mg^{++} partially inhibited it. At concentrations as low as 10^{-6} M a slight effect was still discernible.

The effect of ribonuclease treatment. DNase treatment of a heated mixture of DNA and BSA has been shown to abolish DNA protection, and results in precipitation of the protein.[1] Similar findings were noted when heated RNA-BSA mixtures were subjected to RNase digestion.

The effect of 5' ribonucleotides on heat coagulation of BSA. AMP, UMP, and CMP were unable to prevent the heat coagulation of BSA under conditions identical to those used for the homopolymers.

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Table 1

THE EFFECT OF VARYING THE POLYNUCLEOTIDE:BSA RATIO UPON THE PROTECTION OF BSA AGAINST HEAT COAGULATION. ONE ML BSA-POLYNUCLEOTIDE MIXTURE IN CITRATE BUFFER, 0.0015 $\underline{M}$ pH 5.5 WAS HEATED FOR 10 MIN AT 100° C. TURBIDITY WAS ASSESSED BY INSPECTION, AND FOLLOWING CENTRIFUGATION THE SUPERNATANT WAS ANALYZED FOR BSA AND POLYNUCLEOTIDE CONTENT.

Polynucleotide	Polynucleotide concentration (mg/ml)	BSA concentration (mg/ml)	Wt. ratio BSA: polynucleotide	Turbidity and/or coagulation	Polynucleotide remaining in solution (%)	BSA remaining in solution (%)
Rat liver RNA	1	2.88	2.88	0	n.d.[†]	n.d.
	0.5	2.88	5.8	1+	n.d.	n.d.
	0.25	2.88	11.6	2+	n.d.	n.d.
	0.125	2.88	23	3+	n.d.	n.d.
	0.063	2.88	46	4+	n.d.	n.d.
Poly A	.915	3.25	3.6	0	100	100
	.46	3.25	7.1	1+	86	89
	.23	3.25	14.1	2+	70	74
	.12	3.25	27	4+	0	2
Poly C	1	2.88	2.88	1+[*]	100	98
	0.5	2.88	5.8	2+	89	61
	0.25	2.88	11.6	3+	71	26
	0.125	2.88	2.3	4+	8	0

[*]This Poly C-BSA mixture was slightly turbid prior to heating. There was no visible change in degree of turbidity after heat (see text).

[†]n.d. - not done.
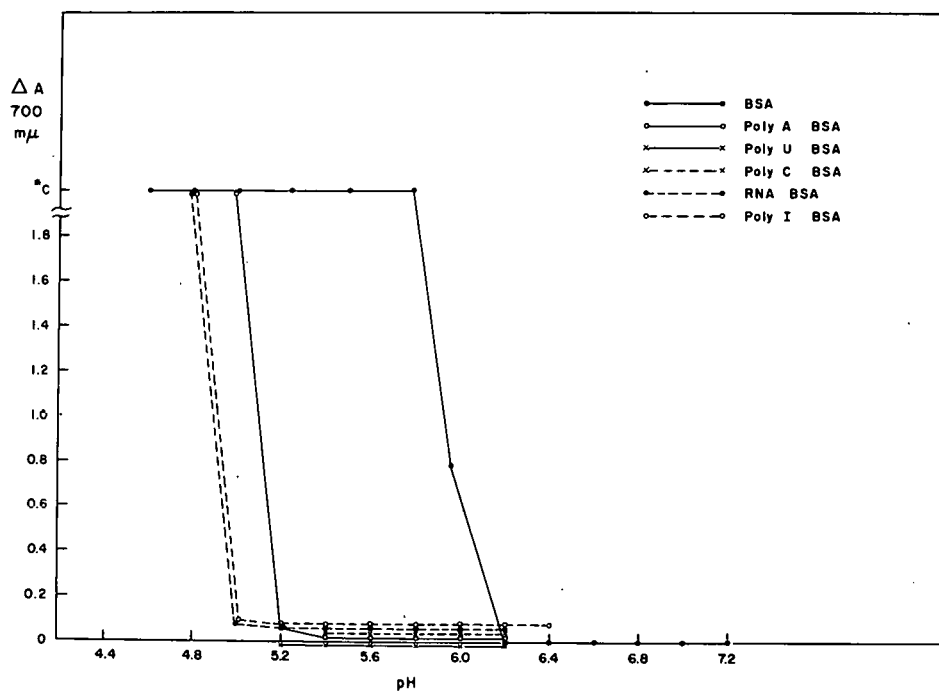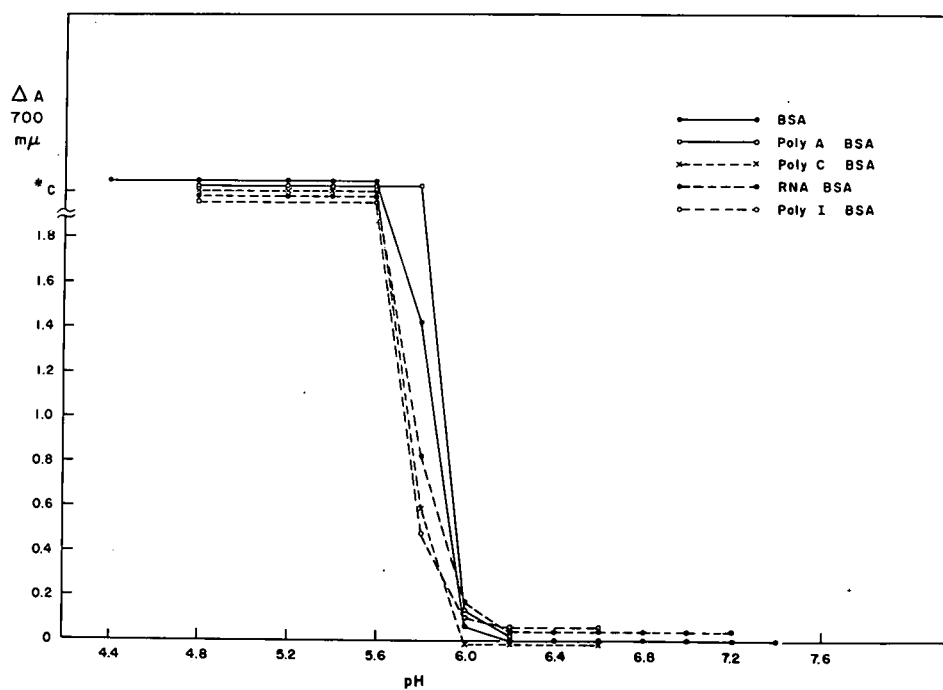
Figure 1. Turbidity changes occurring when BSA alone or mixtures of polyribonucleotides and BSA are heated at 100° C for 10 min. BSA concentration = 1.5 mg/ml. Polyribonucleotide concentration = 0.5 mg/ml. A, Buffer concentration 0.0015 M (pH 4.4 - 6.4 citrate buffer, pH 6.6 - 7.2 phosphate buffer). B, Buffer concentration 0.05 M (pH 4.4 - 6.2 citrate buffer, pH 6.4 - 7.4 phosphate buffer). *Complete coagulation. Coarse nature of coagulum precluded spectrophotometric measurement.

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Table 2

THE EFFECT OF Mg^{++} UPON THE PROTECTION AGAINST THE HEAT COAGULATION OF BSA AFFORDED BY RNA. 1.5 mg BSA, 0.5 mg RAT LIVER RNA IN 1 ml 0.0015 ACETATE BUFFER, pH 5.45. HEATED 10 MIN AT 100° C.

$[Mg^{++}]$	O. D. 700 mμ
10^{-3} M	Complete coagulation*
10^{-4} M	0.265
10^{-5} M	0.087
10^{-6} M	0.057
0	0.023

*Coarse coagulum precluded spectrophotometric measurement.

DISCUSSION

The results reported here, which demonstrate that both natural and synthetic polyribonucleotides can inhibit the heat coagulation of BSA are at variance with those of Carter and Greenstein[10] who found that, whereas DNA could prevent the heat coagulation of egg albumin, yeast RNA was ineffective. Since the four synthetic homopolymers, RNA, and DNA, resemble one another very closely in their behavior towards BSA in solution, it seems reasonable to suspect that it may not be the substituent bases which are crucial in this interaction, but rather the "back bone" of the polynucleotide molecule. Such, indeed, has been the interpretation of other authors[3] who from the clearly electrostatic nature of the interaction between DNA and native BSA, concluded that positively charged groups of the protein were reacting with the negatively charged phosphate groups of the DNA.

The mechanism of the interaction between DNA and BSA which accounts for the inhibition of protein heat coagulation is not as clear-cut. Steiner and Beers, in their recent monograph have been reluctant to accept that complexing is mediated by electrostatic forces exclusively.[6a] Much of the evidence presented here strongly points to electrostatic forces being of importance in the protective effect of polyribonucleotides on BSA heat coagulation. The following papers of this series will deal at greater length with the nature of these heated BSA polynucleotide complexes.

The experiments with RNase treatment of heated BSA-RNA solutions suggest that oligo- or mononucleotides are inadequate for protection. This conclusion is borne out by the observations on the lack of protection of 5' ribonucleotides on BSA heat coagulation. In addition it should be pointed out that the precipitation of BSA following RNase digestion indicates that the protective effect of polyribonucleotides, as in the case of DNA, extends only to heat coagulation, and not to heat denaturation.

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STUDIES ON THE INTERACTION BETWEEN BOVINE SERUM ALBUMIN AND NATURAL AND SYNTHETIC POLYRIBONUCLEOTIDES II. STUDY OF THE INTERACTION BY MEANS OF DENSITY GRADIENT ULTRACENTRIFUGATION[*]

By

S. Yachnin[†]

The previous paper in this series described the protection against the heat coagulation of bovine serum albumin (BSA) afforded by various polyribonucleotides.[1] Immunologic study of heated polynucleotide-BSA mixtures suggested that interaction between the two was strictly limited by conditions of pH and ionic strength.[2] The technique of density gradient ultracentrifugation in a sucrose medium has made possible the elucidation of the nature of complexes formed between BSA and polynucleotide, both with and without heat.

MATERIALS AND METHODS

The method of preparation or commercial source of the materials used have already been described.[1] Unheated polynucleotide-BSA mixtures were studied in citrate buffers of varying concentration and pH; in 0.0015 M acetate buffer, pH 5.5, and in 0.145 M NaCl, 0.015 M phosphate buffer, pH 7.4 (P-S buffer). Heated polynucleotide-BSA mixtures were studied in P-S buffer, and in 0.0015 M citrate, pH 5.5. In order to determine the effect of increases in ionic strength and pH on the heated complexes formed in the latter buffer solution, one-tenth volume of 10 X concentrated P-S buffer was added after the heated mixtures had cooled to room temperature. The ratio of polynucleotide:BSA was kept constant in all experiments (1:3); the usual polynucleotide concentration was 1 mg/ml.

Density gradient ultracentrifugation was carried out in a linear sucrose density gradient of 10 - 40 per cent prepared by means of a mechanical density gradient forming device. 0.2 ml aliquots of BSA, polynucleotide, and BSA-polynucleotide mixture were layered over 4.8 ml sucrose density gradient solution prepared in the same buffer, and all 3 were simultaneously centrifuged in the SW-39, 3-place swinging bucket rotor of the Spinco Model L preparative ultracentrifuge at 38,000 rpm. Following the run, 15 drop fractions were collected by needle puncture of the bottom of the tube. After emptying the tube of all liquid material, very heavy rapidly sedimenting material was recovered from the bottom of the tube (B fraction). Aliquots of each fraction were then analyzed for protein,[3] and for polynucleotide content (UV absorption).

In experiments involving polynucleotide-BSA mixtures heated in 0.0015 M, pH 5.5 citrate buffer, BSA heated alone could not be used as a control since under these conditions it is completely coagulated.[1] In order to be certain that the changes in sedimentation properties of the BSA-polynucleotide complexes formed under these conditions were attributable to association and subsequent dissociation when 10 X concentrated P-S buffer was added to the heated mixtures,

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[*]This report is taken from a paper appearing in Biochim. et Biophys. Acta, 72:578, 1963.

[†]John and Mary Markle Scholar in Academic Medicine.

adequate controls were included. A typical protocol of such an experiment is shown in Table 1.

RESULTS

Unheated polynucleotide-BSA mixtures. Mixtures of polynucleotide and BSA in citrate buffer, 0.0015 M, pH 5.5 (ionic strength 0.005), all showed the appearance of soluble rapidly sedimenting complexes (Fig. 1). If the concentration of the citrate buffer was kept constant (0.0015 M) and the pH raised to 6.5, the formation of soluble complexes was completely abolished in all instances save for Poly I-BSA mixtures (Fig. 2); however the interaction was much weaker than that seen at pH 5.5. If the pH was kept constant at 5.5, and the concentration of the citrate buffer was raised to 0.05 M (ionic strength 0.165), interaction between polynucleotide and BSA was again abolished.

Table 1

A TYPICAL PROTOCOL FOR DETERMINING IF (a) ASSOCIATION OF POLY-
NUCLEOTIDE-BSA OCCURS WHEN THE MIXTURE IS HEATED TOGETHER
IN CITRATE BUFFER 0.0015 $\underline{M}$, pH 5.5 (COMPARE 1 AND 3) AND
(b) IF DISSOCIATION OCCURS WHEN THE pH AND IONIC
STRENGTH OF THE HEATED MIXTURE IS RAISED BY
MEANS OF 10 X CONCENTRATED P-S BUFFER
(COMPARE 1 AND 2). THE REMAINDER
SERVE AS CONTROLS.

Solution heated in citrate buffer, 0.0015 M, pH 5.5	Addition after heating	Sucrose density gradient ultra-centrifugation performed in
1. BSA-polynucleotide	None	0.0015 $\underline{M}$ citrate, pH 5.5
2. BSA-polynucleotide	1/10 volume 10 X concentrated P-S buffer	P-S buffer
3. Polynucleotide	None	0.0015 $\underline{M}$ citrate, pH 5.5
4. Polynucleotide	1/10 volume 10 X concentrated P-S buffer	P-S buffer
5. BSA (heated in P-S buffer)	None	P-S buffer
6. Polynucleotide (heated in P-S buffer)	None	P-S buffer

In the light of the above findings it was not surprising to find a lack of interaction between polynucleotide and BSA in P-S buffer (ionic strength 0.184).

Mg^{++} in low concentration has been found to abolish the protective effect of polynucleotide against the heat coagulation of BSA;[1] 0.5×10^{-3} M Mg^{++} is also sufficient to inhibit soluble complex formation between polynucleotide and BSA.
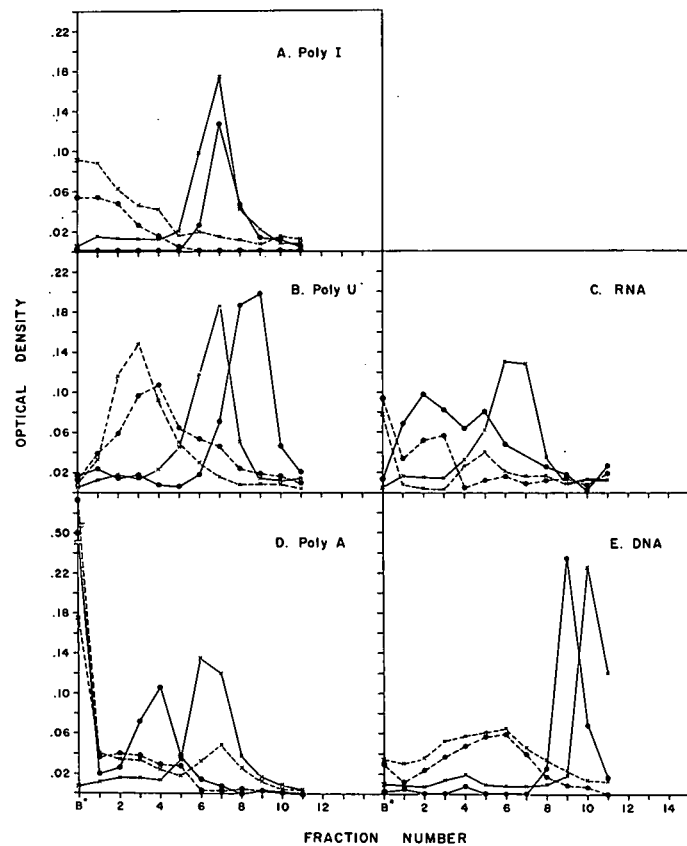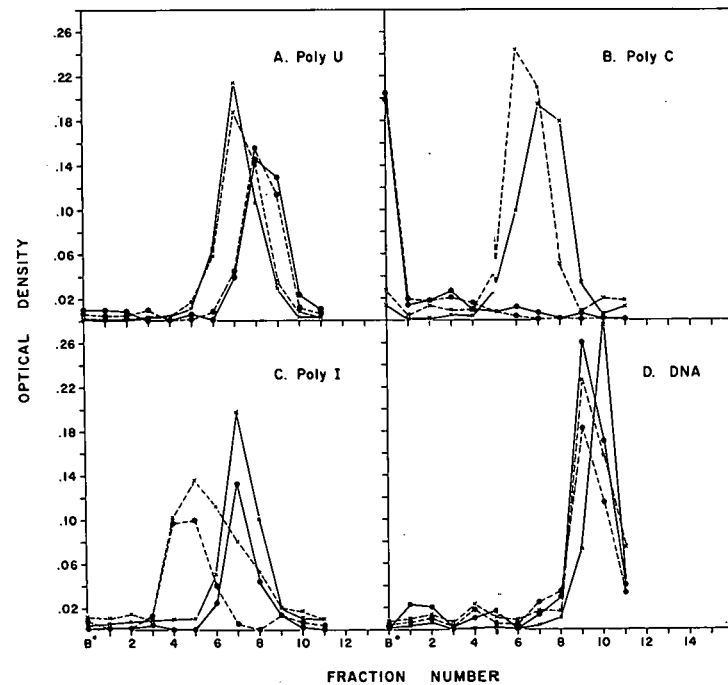
118

Figure 1. Sucrose density gradient ultracentrifugation of unheated BSA-polynucleotide mixtures in 0.0015 M citrate buffer, pH 5.5. Duration of the runs: Poly I - 16 h, Poly U - 16 h, RNA - 16 h, Poly A - 16 h, DNA - 2 h. x - BSA, ●- polynucleotide, —— centrifuged separately, ----- BSA-polynucleotide mixture. *B - bottom fraction (see Methods).

Figure 2. Sucrose density gradient ultracentrifugation of unheated BSA-polynucleotide mixtures in 0.0015 M citrate buffer, pH 6.5. Duration of the runs: Poly U - 16 h, Poly C - 15.5 h, Poly I - 16 h, DNA - 2 h. x - BSA, ●- polynucleotide, —— centrifuged separately, ----- BSA-polynucleotide mixture. *B - bottom fraction (see Methods).

<u>Heated polynucleotide-BSA mixtures</u>. Polynucleotide-BSA mixtures heated in P-S buffer show no evidence of complex formation (Fig. 3). Rapidly sedimenting soluble complexes are formed when polynucleotide and BSA are heated together in citrate buffer 0.0015 M, pH 5.5. However, these complexes (including those formed between DNA and BSA) are easily dissoci-
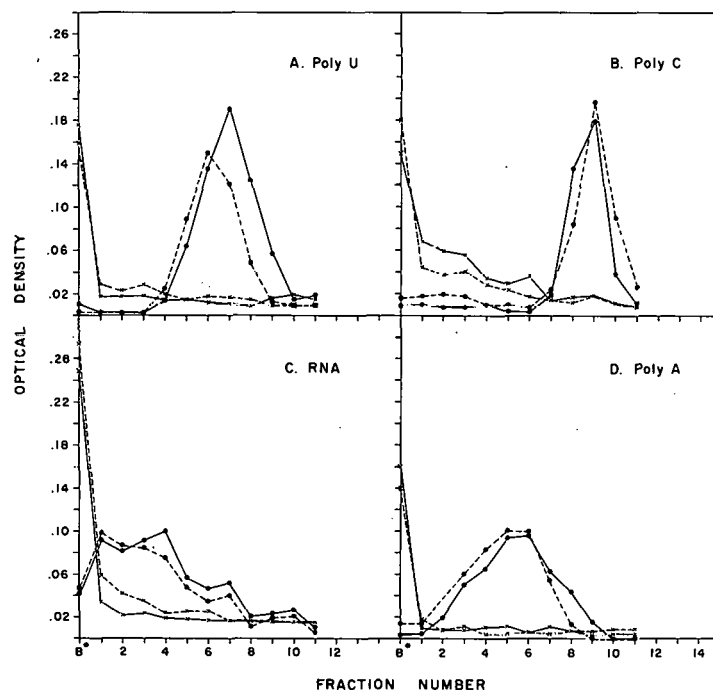
Figure 3. Sucrose density gradient ultracentrifugation of heated BSA-polynucleotide mixtures in P-S. Note the lack of complex formation. Duration of the runs: Poly U - 16 h, Poly C - 5 h, RNA - 14.5 h. Poly A - 16 h. x - BSA, ● - polynucleotide, ———— heated and centrifuged separately, - - - - - BSA-polynucleotide mixture. *B - bottom fraction (see Methods).

ated by raising the ionic strength and pH of the solution to 0.184 and 7.4 respectively (Fig. 4). It should be noted that no protein having the sedimentation properties of native BSA can be found either before or after dissociation occurs; thus the protective effect of polynucleotide or BSA extends only to heat coagulation but not to aggregation.

DISCUSSION

The conditions under which soluble complex formation occurs between polyribonucleotides and native BSA are strictly limited by the ionic strength and pH of the solution, and resemble very closely those previously described for DNA and native BSA.[4] Inhibition of the formation of soluble complexes between the native protein and polynucleotide results in the abolition of the protective effect of polynucleotide against the heat coagulation of the protein.[1]

Polynucleotide-BSA complexes persist after heat. Electrostatic forces appear to play a major role in the formation of complexes between polynucleotides and heated as well as native
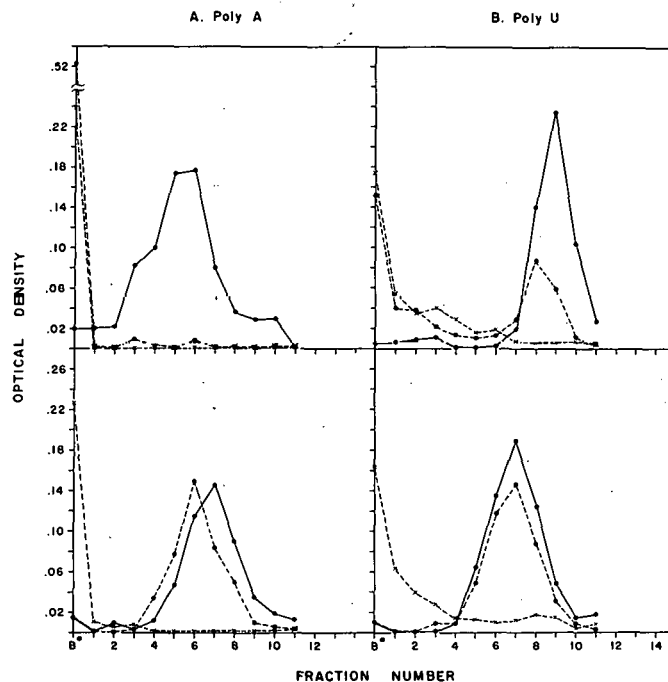
Figure 4. The dissociation of heated BSA-poly-nucleotide complexes formed in 0.0015 M citrate buffer, pH 5.5, by raising the ionic strength and pH of the solution. The upper figures in each column represent the BSA-polynucleotide mixtures (and polynucleotide alone) heated and run in the citrate buffer. The lower figures represent the same heated solutions following the addition of 1/10 volume 10 X concentrated P-S buffer. The latter runs were performed in a P-S buffer sucrose density gradient. Note that following disso-ciation, no protein with the sedimentation proper-ties of native BSA (usual peak fraction 6 or 7) is seen. Duration of runs: Poly A - 15.75 h, Poly U - 16 h. x - BSA, ● - polynucleotide, ——— cen-trifuged separately, - - - - - BSA-polynucleotide mixture. *B - bottom fraction (see Methods).

BSA. The effects of Mg^{++} ion, which in low concentration abolishes the protective effect of poly-nucleotide on BSA heat coagulation and prevents the formation of soluble complexes between na-tive BSA and polynucleotide, are pertinent to this argument. Felsenfeld[6] has shown that Mn^{++} and Mg^{++} react stoichiometrically with the phosphate groups of Poly A, Poly U, and denatured DNA, and Steiner and Beers conclude that "it is very highly probable that the phosphates are the only important binding sites."[7] This inhibitory effect of Mg^{++} on polyribonucleotide-BSA interactions resembles that of Mg^{++} upon the binding of proflavine by DNA,[8] where electrostat-ic forces play a major role, and in both instances the negatively charged phosphate groups serve as the major site of interaction.

Zubay and Doty[5] suggested that binding between DNA and heated BSA is mainly due to hy-drogen bonds, basing this conclusion upon the fact that addition of 8 M urea to heated DNA-BSA complexes resulted in a drastic fall in light scattering. Apart from the fact that 8 M urea solu-

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tion raises the dielectric constant and thereby may weaken electrostatic interactions, there is growing evidence that urea denaturation may proceed by mechanisms other than hydrogen bond disruption.[9-11] In a note immediately following the Greenstein and Hoyer paper[12] it was remarked that the patterns obtained on electrophoresis or ultracentrifugation of heated DNA-BSA mixtures were dependent upon the salt content of the medium. The results presented in this paper agree with the latter observation. Complexes formed between heated BSA and polynucleotide are easily dissociated by raising the ionic strength and pH of the solution. Thus, while the evidence presented does not rule out the possibility of the existence of hydrogen bonds in heated polynucleotide-BSA complexes, electrostatic forces can be, by themselves, stability determining for such complexes, as well as for polynucleotide-BSA complexes formed without the application of heat.

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SYNTHESIS OF POLYPSEUDOURIDYLIC ACID BY POLYNUCLEOTIDE PHOSPHORYLASE[*]

By

L. Sasse,[†] M. Rabinowitz, and I. H. Goldberg

The reversible polymerization of ribonucleoside diphosphates was first demonstrated by Grunberg-Manago and Ochoa[1] in extracts of Azotobacter vinelandii and later by Littauer and Kornberg[2] in Escherichia coli and by Beers,[3] and Olmstead[4] in Micrococcus lysodeikticus. Homopolymers of adenylic, uridylic, cytidylic and inosinic acids were readily formed, as were mixed polymers of two or more of the ribonucleotides in various ratios. However, polyguanylic acid formation required an oligonucleotide primer, as shown by Singer et al.[5,6] The addition of di-, tri- or tetranucleotides with a free 3'-hydroxyl function was necessary for polyguanylic acid formation, when purified enzyme systems were used. The oligonucleotides were incorporated into the polymers, serving as starting points for chain proliferation. Whereas an absolute primer requirement for poly G formation was found, primer served only to reduce the latent period in poly A, poly U or poly ribothymidylic acid synthesis. More recently Fresco and Su[7] reported poly G synthesis without use of primer.

Certain substituted nucleoside diphosphates are also effectively polymerized by polynucleotide phosphorylase. Michelson et al.[8] showed that substitution of hydrogen at C-5 in uracil by bromine, chlorine or iodine did not diminish the activity of the nucleoside diphosphates with respect to polymerization, Pi release, or Pi^{32}-nucleoside diphosphate exchange. These halogenated derivatives are analogues of ribothymidine diphosphate. In addition, the diphosphates of 2-thiouridine[9] and 5-fluorouridine[10] can serve as substrates for polynucleotide phosphorylase. Several other diphosphates of substituted uridine and guanosine compounds are polymerized by polynucleotide phosphorylase; others are inactive as substrates, and some act as inhibitors of the enzyme.[11-14]

Pseudouridine triphosphate substitutes completely for UTP in the bacterial and mammalian RNA polymerase systems,[15-17] and pseudouridine diphosphate glycosyl compounds are enzymatically synthesized by yeast extract[18] and participate in glycosyl transfer and transformations.[19] It might therefore be expected that ψUDP would also be an active substrate for polynucleotide phosphorylase. This paper describes the synthesis of polypseudouridylic acid from ψUDP by

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polynucleotide phosphorylase. Primer pApApA greatly stimulates the rate of polymerization, but an absolute requirement is not shown when relatively impure enzyme preparations are used.

METHODS

Inorganic phosphate was determined by the method of Fiske and SubbaRow.[20] ADP and UDP were obtained from Schwarz Bio-Research Inc., 3 x recrystallized pancreatic ribonuclease from Worthington Biochemical Corp., and Crotalus adamanteus venom from Ross Allen Reptile Farm. Snake venom diesterase was purified by the method of Koerner and Sinsheimer[21] and was free of 5'-mononucleotidase activity.

Whatman 3MM paper was used for descending chromatography. Solvent system I: ethanol, 0.5 M ammonium acetate pH 3.8 (5:2 vol/vol). Solvent system II: isopropanol, 0.1 M sodium borate, pH 9.1 (7:3 vol/vol). Paper electrophoresis in 0.025 M citrate buffer, pH 5.4 was run at 250 volts for 18 h.

Preparation of pseudouridine diphosphate. Pseudouridine, isolated from urine, was converted to its isopropylidene derivative and then chemically phosphorylated with ^{32}P-labeled or non-radioactive 2-cyanoethylphosphate.[22,23] ψUTP was synthesized enzymatically from the 5'-mononucleotide with a yeast enzyme fraction.[22,23] Rabbit muscle myosin ATPase[24] was used to remove the terminal phosphate of ψUTP. The reaction mixture consisting of 2.5×10^{-3} M ψUTP, 0.1 M glycine pH 9.1, 10^{-3} M $CaCl_2$ and 0.1 mg/ml myosin was incubated for 10 min at 23°. The ψUDP, which was quantitatively produced from ψUTP, as shown by paper electrophoresis, was isolated by column chromatography on DEAE-cellulose (carbonate) using TEAC as eluent. ψUDP appeared at about 0.14 M TEAC when an exponential gradient from 0.04 M (500 ml mixing chamber) to 0.2 M TEAC (1000 ml reservoir) was used. Preparation of 5-FUDP from 5-FUTP was analogous to that described for ψUDP. 5-FUTP was prepared as described.[23]

Enzyme preparations.

Preparation A — Polynucleotide phosphorylase was prepared from M. lysodeikticus by the method of Beers,[3] the purification being carried through the acetone fractionation step. Specific activity was 3.2 μmoles Pi released from UDP in 15 min per mg protein.

Preparation B — Another preparation of polynucleotide phosphorylase containing less nucleic acid was made using DEAE-cellulose chromatography. Lysis of acetone dried M. lysodeikticus cells was performed according to Beers[3] and carried through the 2 ammonium sulfate precipitations. The precipitate was dissolved in 0.02 M Tris, pH 8.1, dialyzed against the buffer for 24 h, and applied to a DEAE-cellulose column which had been washed with 0.02 M Tris, pH 8.1, 0.02 M KCl. Elution was effected with an exponential gradient using a mixing chamber containing 125 ml of 0.02 M KCl, 0.02 M Tris, pH 8.1, and a reservoir containing 0.14 M KCl, 0.02 M Tris 8.1. The enzyme activity appeared in 120 - 160 ml. The active fractions, assayed by Pi release, were pooled, and concentrated by precipitation with ammonium sulfate added to 0.75 saturation. The precipitate was dialyzed against 0.05 M Tris, pH 8.1. The A_{280}/A_{260} was 1.68. Specific activity was 5.6 μmole Pi released from UDP in 15 min per mg protein.

Assays. Enzyme preparations were assayed by Pi release and [32]Pi-nucleoside diphosphate exchange as described by Singer and Guss,[6] and by polymer formation. Standard polymer formation was determined using ^{32}P-labeled nucleoside diphosphate. The reaction mixture contained 5×10^{-3} M $MgCl_2$, 6.5×10^{-2} Tris, pH 9.5 and 1×10^{-2} M UDP^{32} or ψUDP^{32} with 3×10^{-4} M pApApA added as indicated. The reaction was stopped by boiling for 2 min, the mixture centri-

fuged, and a suitable aliquot of the supernatant fluid applied to Whatman 3MM paper. The radio-activity at the origin was determined using a Tracerlab 3NA end-window Geiger-Muller tube, and an Atomic scaler. After developing the chromatogram in solvent I, the radioactivity remaining at the origin, corrected for decay, represented conversion to polynucleotide.

Isolation of polynucleotide. For characterization of polynucleotide, labeled and non-radio-active products were isolated after incubation under standard conditions for 120 min at 37° as follows: The reaction was terminated and protein removed by phenol treatment.[25] Extractions were done at 4° for 8 min, 2 min, and 2 min, using an equal volume of freshly distilled phenol saturated with 0.02 M phosphate buffer pH 7.6. The phenol was removed by repeated other extractions, and the polynucleotide precipitated with two volumes of absolute ethanol. The precipitate was dissolved in a small volume of 0.05 M NaCl, 0.02 M Na citrate, and dialyzed 24 h against two changes of a thousand volumes of 0.05 M NaCl, and 48 h against distilled H_2O.

RESULTS

Synthesis of poly ψU was found with enzyme preparations A and B. With both enzyme preparations, the rate of synthesis of poly ψU was markedly increased by the addition of primer pApApA (Fig. 1). Similar results were obtained when Pi release was followed (Fig. 2). Some ac-
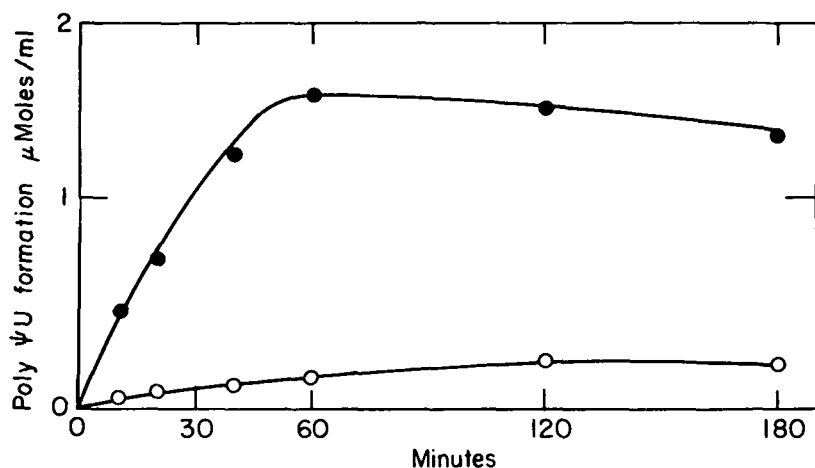

Figure 1. Effect of pApApA upon poly ψU synthesis. The 0.1 ml reaction mixture contained 6.7×10^{-2} M Tris pH 9.5, 5×10^{-3} M $MgCl_2$, 1×10^{-2} M ψUDP^{32} (specific activity 6×10^4 cpm/μmole) and 230 μg polynucleotide phosphorylase preparation A. o—o without pApApA, ●—● with 3×10^{-4} M pApApA. Incubation was at 23°. 10 μl aliquots were removed at the time indicated, diluted with 20 μl water and heated to 100° for 1 min. After centrifugation, 20 μl of supernatant fluid was applied to Whatman 3MM paper, and chromatography performed in solvent system A. Polymer formation was determined as described in Methods.

tivity was present, however, in the absence of primer and polymer formation was more affected by primer than was Pi release. The stimulation by primer of poly U formation was less pronounced. These effects contrast to the obligatory primer requirement in polyguanylic acid synthesis found by Singer et al.[5,6]
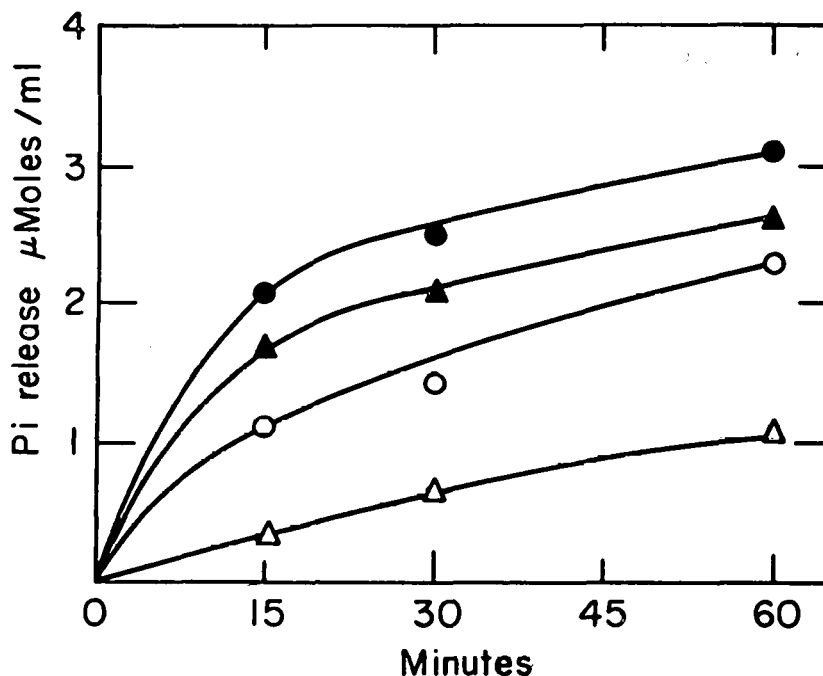
Figure 2. Effect of pApApA on Pi release from ψUDP and
UDP. Reaction conditions were identical to those used for
Figure 1. Preparation A enzyme was used △——△ ψUDP as
substrate, o——o ψUDP as substrate with 3×10^{-4} M
pApApA primer added, ▲——▲ UDP as substrate, ●——● UDP
was substrate and 3×10^{-4} M pApApA primer added. 10 μl
aliquots were used for Pi determination,[20] the method being
modified so as to take final readings in a volume of 1.0 ml
with the Beckman DU spectrophotometer.

Relative rates of polymer synthesis from ψUDP and UDP precursors varied with different
enzyme preparations, and with the aging of the preparations. Because of enzyme impurities and
side reactions, and possible differences in the conditions optimal for the two substrates, a firm
statement of relative reaction rates cannot be made. With most preparations, however, rates
with ψUDP were 2 to 3 times slower than with UDP.

Poly ψU is also synthesized with RNA polymerase of E. coli using ψUTP as substrate and
calf thymus DNA as primer.[23] Its rate of synthesis is identical with that of poly U from UTP.
Although synthesis of poly ψU by the E. coli RNA polymerase system is considerably slower
than that with polynucleotide phosphorylase, it seemed desirable to verify the fact that with the
preparations of polynucleotide phosphorylase used, the nucleoside diphosphate rather than the
triphosphate was the true precursor. Figures 3A and 3B show radioautograms of chromatograms
developed in solvent system I, demonstrating poly ψU formation from ψUDP^{32}, but not from
ψUTP^{32}.

ψUDP was also an active substrate for polynucleotide phosphorylase when assayed by ^{32}Pi-
ψUDP exchange. Table 1A shows the relative rates of ^{32}Pi-exchange with ψUDP, UDP, 5-FUDP
and ADP. The reaction products were verified as the radioactive nucleoside diphosphates by pa-
per electrophoresis. In these experiments the exchange with ψUDP was considerably less rapid
than with UDP, 5-FUDP, or ADP. It is seen in Table 1B that the addition of primer pApApA did
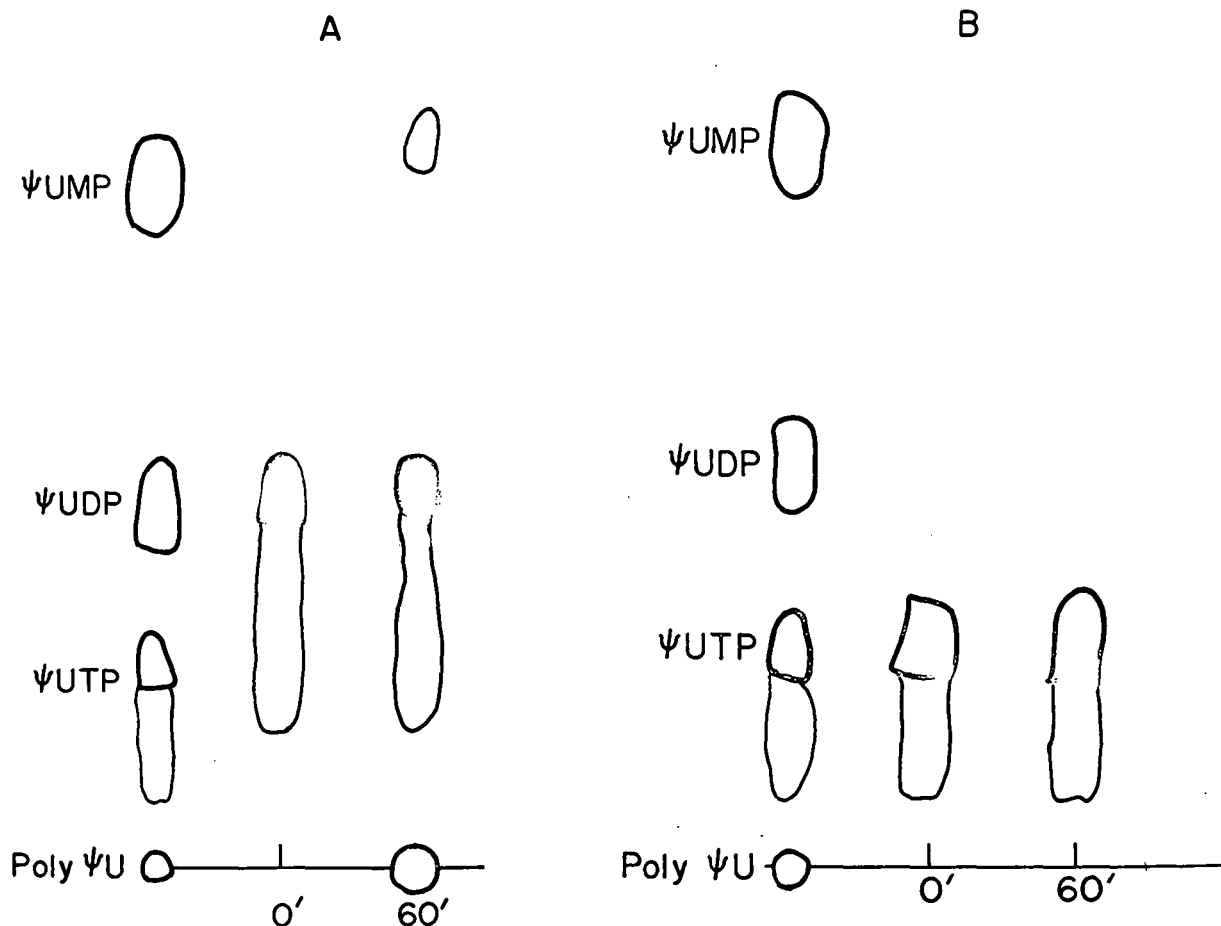not appreciably affect the exchange rates. 5-FUDP was found to be polymerized at rates similar

Figure 3. Autoradiography of a paper chromatogram developed in solvent system I showing: (A) poly ψU formation from ψUDP^{32}. (B) No polymer formation from ψUTP. Standard polymerization reaction conditions were used including 3×10^{-4} M pApApA. In B, 10^{-2} M ψUTP^{32} was substituted for ψUDP^{32}, and 10^{-2} M phosphoenolpyruvate and 10 μg phosphoenolpyruvate kinase were added per ml of reaction mixture. The material remaining at the origin (ultraviolet absorbing and ^{32}P-labeled) could be eluted with 0.1 M sodium chloride and upon hydrolysis with 0.3 M KOH yielded 2'(3')ψUMP.

to or greater than that for UDP, which contrasts with the negligible homopolymer formation found with 5-FUTP and RNA polymerase primed by calf thymus DNA[23].

The isolated poly ψU was free of contaminating mononucleotides as shown by paper chromatography in solvent system I, in which all the radioactivity and ultraviolet absorbing material remained at the origin. The ultraviolet absorption spectrum (Fig. 4) of the polymer displays the alkaline bathochromic shift characteristic of pseudouridine derivatives. A shoulder is present at 260 mμ at pH 13.0 which is probably due to incorporated primer pApApA. The λ max, and λ min in acid and alkali are similar to those reported for pseudouridine[26,27] and for chemically prepared poly ψU,[28] containing both 2' → 5' and 3' → 5' phosphodiester linkages. Of particular note are the almost identical extinctions at λ max at pH 7 and 13 of the enzymatically prepared polymer. This contrasts to the 20 per cent decrease in extinction at λ max at pH 13 of the 5'-nucleotide derivatives of pseudouridine (ψUMP, ψUDP, ψUTP, ψUDP-glucose),[18,22] but is similar to the spectra of the 2'(3') phosphate of pseudouridine and of the nucleoside itself.[26] In poly

Table 1

NUCLEOSIDE DIPHOSPHATE - Pi^{32} EXCHANGE

Experiment	Nucleotide	cpm	In nucleoside diphosphate
		10 min	20 min
A	ADP	3,300	4,949
	UDP	1,096	2,095
	ψUDP	330	657
	5-FUDP	2,623	3,089
		15 min	
B	UDP	10,473	
	UDP + pApApA	10,185	
	ψUDP	2,556	
	ψUDP + pApApA	2,553	

Experiment A. The 25 μl incubation mixture contained 0.1 $\underline{M}$ Tris pH 8.6, 3 x 10^{-3} $\underline{M}$ (P^{32}) K$_2$HPO$_3$ specific activity 1.8 x 10^5 cpm/μmoles, 5 x 10^{-3} $\underline{M}$ MgCl$_2$, 3 x 10^{-4} $\underline{M}$ EDTA, 4 x 10^{-3} $\underline{M}$ nucleoside diphosphate and 31 μg polynucleotide phosphorylase, preparation A. Incubation was at 37°.

Experiment B. The 50 μl incubation was the same as in Experiment A except that 75 μg of enzyme was used and 3 x 10^{-4} pApApA was added where indicated. The specific activity of the (P^{32}) KHPO$_4$ was 4.2 x 10^5 cpm/μmole. Incubation was at 23° for 15 minutes.

ψU the relative absorptions at λ max in neutral pH and in alkali are affected by several variables. The presence of adenylic acid incorporated as primer would result in a significant decrease in optical density at λ max in alkali. This would be counterbalanced by any hyperchromic effect at 286 mμ in alkali due to the helix-coil transition. These opposite effects are probably of similar magnitude and would cancel each other out. One might then expect a further decrease in extinction in alkali as has been noted for the 5'-mononucleotides.[18,22] This, however, is not found. The decrease in extinction at λ max in alkali of the pseudouridine-5'-phosphate may be due to interreaction of the 5'-phosphate with the N$_3$ function of the pyrimidine ring. Molecular models show that this is possible in all the 5'-nucleotides so far studied: ψUMP, ψUDP, ψUTP, ψUDP-glucose. This cannot be shown for 2'(3')-ψUMP, and it may be that there is also hindrance to interreaction of the phosphate group with the ring nitrogen in the more rigid structure of poly ψU.

The polymer was shown to be polypseudouridylic acid possessing the natural C 3' $\rightarrow$ C 5' phosphodiester linkage, by its characteristic chemical and enzymatic reactions. Incubation of ^{32}P-labeled polymer with pancreatic RNase yielded 3'-ψUMP^{32}, which was identified by paper chromatography in solvent systems I and II, and by absence of reaction with the 5'-mononucleotidase of snake venom. The product released after treatment of the polymer with purified snake venom diesterase was characterized as the 5'-ψUMP, by chromatography in solvent systems I and II, and by release of Pi^{32} on treatment with 5'-mononucleotidase. Alkaline hydrolysis of the
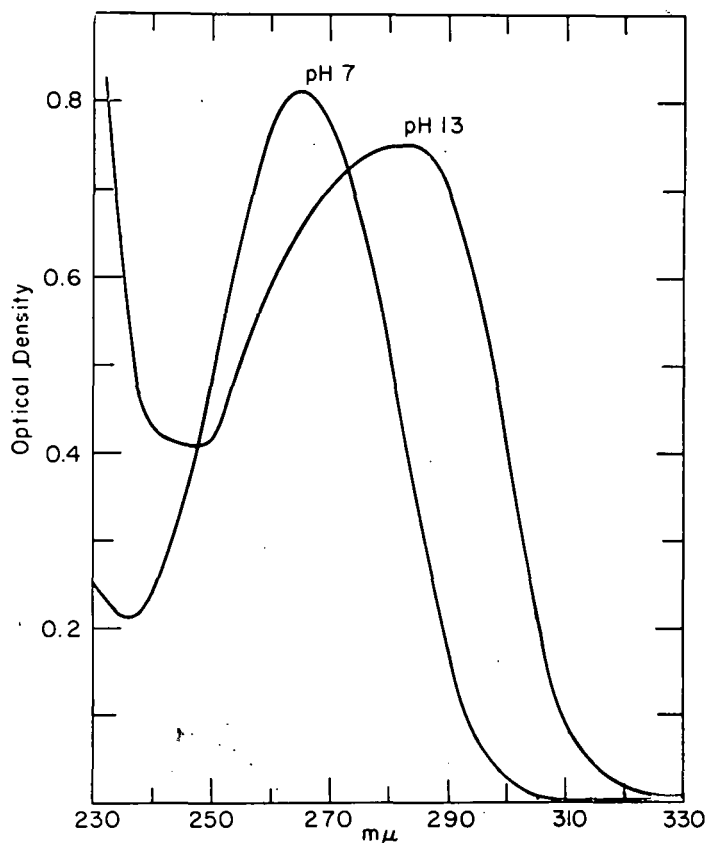
Figure 4. Ultraviolet absorption spectra of poly-
pseudouridylic acid at pH 7 and at pH 13. The
spectra were recorded with a Cary Recording
Spectrophotometer Model 14. Enzyme prepara-
tion B was used to prepare the polymer using
standard reaction conditions, containing 3×10^{-4}
M pApApA. The polymer was isolated as de-
scribed in Methods.

polymer with 0.3 N NaOH at 37° for 18 h yielded 2'(3')-ψUMP. Phosphorolysis by polynucleotide
phosphorylase produced ψUDP. The small amount of ψUMP formed in this reaction is presum-
ably due to contaminating phosphatases.

DISCUSSION

Pseudouridine diphosphate is a substrate for homopolymer formation by polynucleotide
phosphorylase. With partially purified enzyme preparations from M. lysodeikticus the addition
of primer pApApA was necessary for optimal rates of polymer synthesis. Even in the presence
of primer oligonucleotide, however, the rate of poly ψU formation was usually not as rapid as
that of poly U. Pi^{32}-nucleoside diphosphate exchange rates also were slower with ψUDP than
with UDP. In contrast with polymer formation, exchange rates were not affected by primer with
the enzyme preparations used. Singer and Guss[6] however have reported parallel stimulation of
homopolymer synthesis and of exchange rates by pApApA with certain preparations of polynu-
cleotide phosphorylase. These effects were remarkably sensitive to pH, and to small changes in

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substrate and metal concentrations. The disassociation of the primer effect on the synthetic and exchange reactions observed in this report, has been noted by others using a variety of enzyme preparations.[5,29-31]

It is apparent that reaction rates and properties of polynucleotide phosphorylase are exquisitely sensitive to incubation conditions and differences in enzyme preparations. Since systematic exploration of conditions optimal for ψUDP polymerization was not performed, conclusions regarding relative substrate efficiency cannot be made. In some reactions of pseudouridine nucleotides, the rates of reaction are slower than with uridine counterparts. This has been observed with the enzymatic reactions of pseudouridyl glycosyl compounds.[18,19] In contrast, is the efficient and complete substitution of ψUTP for UTP in RNA synthesis catalyzed by RNA polymerase.[15-17,19,23] It is difficult to say into which group the reaction of ψUDP with polynucleotide phosphorylase falls.

The role of primer oligonucleotides on homopolymer synthesis has not been clearly defined. The ability to demonstrate poly G formation in the absence of primer, using conditions of substrate dilution and high enzyme concentration, has led Fresco and Su[7] to suggest that the function of primer is to weaken interreaction of poly or oligonucleotide strands so as to prevent formation of multipolymers which may inhibit the enzyme. Such a mechanism would allow exchange to continue in the absence of primer from the end of the aborted oligonucleotide. Alternate synthesis and phosphorolysis under these circumstances, would not be associated with significant polymerization. The highly ordered structure of guanine oligonucleotide[32] would support such a proposal. In preliminary experiments poly ψU has been shown to form a helix with itself as well as with polyadenylic acid.[33] The possible relationship of these findings to the greater effect of primer on poly ψU formation than on poly U synthesis remains to be elucidated.

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INHIBITION OF RNA POLYMERASE BY 6-AZAURIDINE TRIPHOSPHATE[*]

By

I. H. Goldberg,[†] and M. Rabinowitz

6-azauridine is converted into its 5'-mononucleotide in mammalian[1-3] and microbial[4-6] systems. Handschumacher and Pasternak have demonstrated the competitive inhibition of oroti-dylic acid decarboxylase derived from several sources by exceedingly small amounts of 6-azauridine-5'-phosphate[3,7,8] while the diphosphate and triphosphate were relatively ineffective.[8] This work combined with impressive evidence obtained from in vivo studies[1-3,5] appears to provide a mechanism for the action of azauridine, at least in mammalian cells. Although mammalian cells appear to be defective in the formation of di- and triphosphates of azauridine,[3,7] and unable to incorporate the analog into their nucleic acid,[3] the formation of these compounds and minor incorporation into RNA has been found in bacterial systems.[8,9] Furthermore, 6-azauridine-5'-diphosphate at relatively high concentrations inhibits the exchange reaction with orthophosphate catalyzed by E. coli polynucleotide phosphorylase.[10]

In the course of experiments on the effect of uridylic acid analogues on the formation of RNA by the DNA-directed RNA polymerase of E. coli, it was found that 6-azauridine triphosphate (aza-UTT) would not substitute for UTP in the DNA-dependent pyrophosphate-nucleoside triphosphate exchange reaction catalyzed by this enzyme. Similarly, Kahan has shown this compound not to be utilized in the synthetic reaction.[11] In addition, we have found that aza-UTP acts as an inhibitor of both the synthetic and exchange reaction with RNA polymerase.

E. coli RNA polymerase was prepared and the synthetic reaction assayed as described by Chamberlin and Berg.[12] [^{32}P]UTP, synthesized as previously described,[13] was used as the proximally labeled incorporating nucleotide. Radioactive pyrophosphate (^{32}PP$_i$) was prepared according to Jones et al.,[14] and the incorporation of radioactivity into the terminal pyrophosphate position of nucleoside triphosphates was determined as already described.[15] All incubations were carried out for 15 min at 37°. Aza-UTP, aza-UDP, and aza-UMP were the gifts of the Cancer Chemotherapy National Service Center, National Institutes of Health, Bethesda, Maryland. Calf thymus DNA was obtained from Sigma Chemical Co., St. Louis, Mo. and the naturally-occurring deoxyadenylate-thymidylate copolymer of crab testis (Cancer borealis) which contains only a small amount of deoxyguanylate and deoxycytidylate and consists of almost perfectly alternating deoxyadenylate and thymidylate[16] (crab-dAT) was a gift of Dr. N. Sueoka, presently at Princeton University.

The pyrophosphate-nucleoside triphosphate exchange reaction dependent on crab-dAT (or the dAT synthesized by Kornberg's DNA polymerase) has been shown to be maximal when UTP alone was included in the reaction.[15] ATP was not required for the exchange with UTP and while it could participate in the exchange in the presence of UTP, when used alone exchange was minimal. Inasmuch as this appeared to be a UTP specific reaction (GTP or CTP alone did

not participate significantly in the exchange), it appeared worthwhile to test this for the effect of aza-UTP. As seen in Figure 1, the $^{32}PP_i$-UTP exchange was significantly inhibited by aza-UTP while aza-UDP and aza-UMP were less effective. The concentrations of nucleotides used
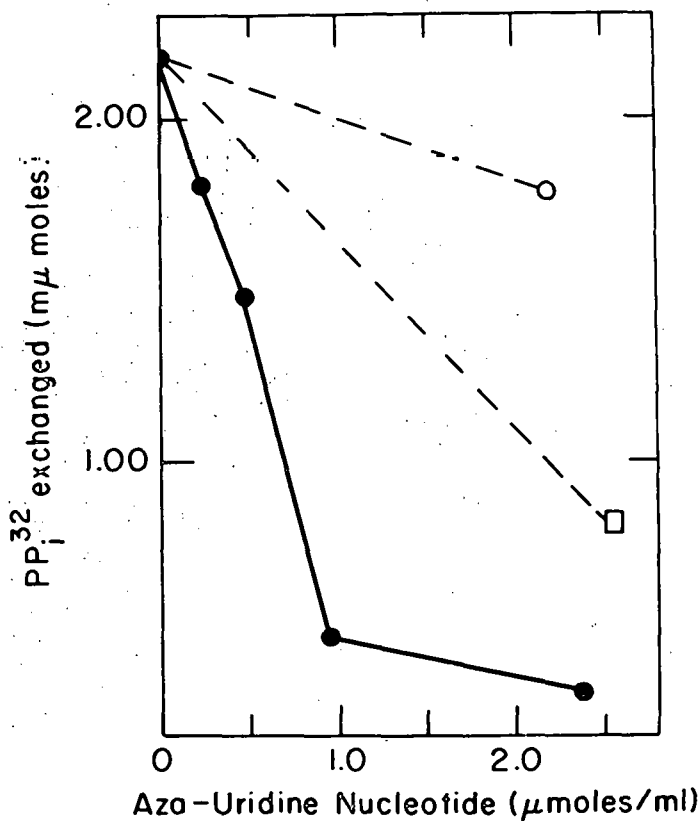

Figure 1. Inhibition by 6-azauridine nucleotides of $^{32}PP_i$-UTP exchange catalyzed by RNA polymerase and crab-dAT. Each tube contained 4 μmoles of Tris buffer, pH 7.8; 0.8 μmoles of MgCl$_2$; 0.16 μmoles of potassium $^{32}PP_i$ (5 x 10^6 counts/min/μmole); 1.0 μmoles of mercaptoethanol; 0.2 μmoles of UTP; 0.0086 O.D. units (260 mμ) of crab-dAT; and 1.64 μg enzyme protein in 0.10 ml final volume. The incubation was stopped and assayed as noted.[15] The concentration of 6-azauridine nucleotide was varied as indicated. o - . - o aza-UMP; □ - - □ aza-UDP; ● - - ● aza-UTP. The $^{32}PP_i$-UTP exchange is expressed on the basis of a 1.0 ml reaction. In the absence of added crab dAT the exchange was only 5 per cent that of the complete system.

in these experiments were such that the optimal ratio of nucleotide to Mg^{++} was not exceeded. Accordingly the inhibitory effects described here can not be ascribed to unfavorable alteration in the reaction conditions. Similarly, as shown in Figure 2, aza-UTP interfered with the incorporation of label from [^{32}P]UTP into RNA catalyzed by the RNA polymerase. The lesser but significant effect of aza-UDP may be due to contaminating nucleotide transphosphorylases.
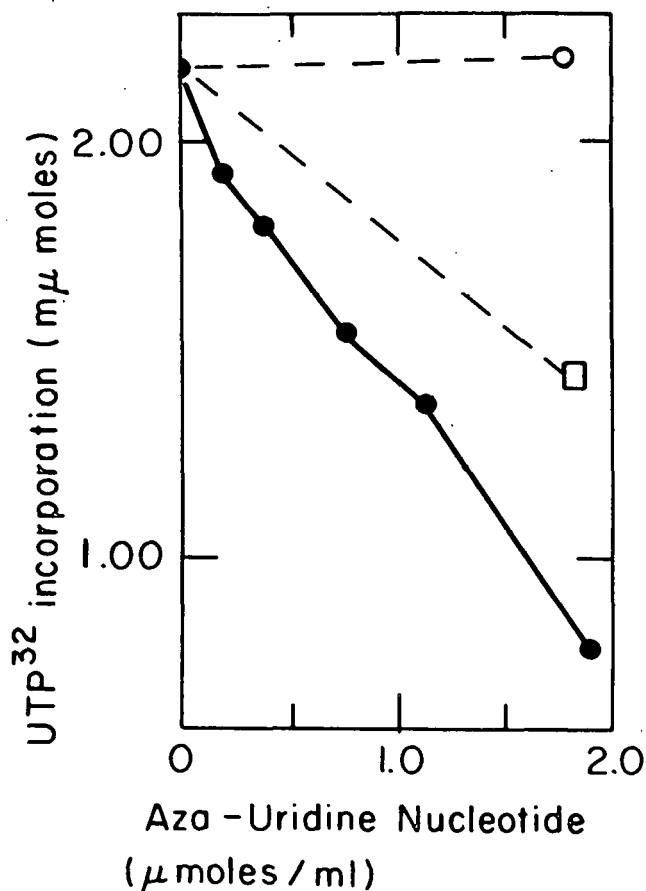
Figure 2. Inhibition by 6-azauridine nucleotides of [^{32}P] UTP incorporation into RNA catalyzed by RNA polymerase and crab-dAT. Reaction conditions are according to Chamberlin and Berg[12] except that each tube contained 0.2 μmoles each of [^{32}P] UTP (0.8 x 10^6 counts/min/μmole) and ATP as the only nucleoside triphosphates, 0.0086 O.D. units (260 mμ) of crab-dAT, and 4.1 μg enzyme protein in 0.25 ml final volume. The concentration of 6-azauridine nucleotide was varied as indicated. o - - o aza-UMP, □ - - □ UDP, ● - - ● aza-UTP. In the absence of added crab-dAT incorporation was only 2 per cent that of the complete system.

While it appeared from the above experiments that the 6-azauridine analogue might be interfering specifically with utilization of UTP in these reactions, other experiments using calf thymus DNA as primer demonstrated the inhibition of ^{32}PP$_i$ exchange with ATP by aza-UTP. This may be related to the finding of Skoda et al.[10] who found inhibition by aza-UDP of both UDP- and ADP-P$_i$ exchange catalyzed by polynucleotide phosphorylase.

The concentration of analogue required to produce the described inhibition of the RNA polymerase and polynucleotide phosphorylase are much higher than needed for interference with the orotidylic acid decarboxylase. Furthermore, the nonspecific nature of the inhibitory effect in the former instances argues against these being prime sites for interference of RNA synthesis or

metabolism by azauridine nucleotides. Nevertheless, when present at such concentrations at least in some systems (notably microorganisms), such activities of 6-azauridine nucleotides may have some importance.

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A SMALL ANIMAL SCANNING SYSTEM[*]

By

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In biological research it is often necessary to determine the distribution of some substance in an experimental animal. In recent years such studies have been facilitated by the use of materials labeled with radioisotopes. The procedure used depends on the nature of the radiation emitted by the tracer and the required spatial resolution. When applicable, autoradiographic techniques[1] performed on excised tissue sections yield distribution information at the cellular level. Where such detail is not required, distribution at the organ level is determined by well counter measurement of activity in tissue samples. In addition, profile scanning[2] with a slit collimator has been used to determine the regional distribution of radioactivity.

A technique which has received little attention for animal studies is the use of radioisotope scanning systems similar to those employed in diagnostic medicine for the mapping of activity in the thyroid, brain, liver, etc.[3] This is partly because in designing systems for human use the need to minimize radiation dosage to the patient gives high counting efficiency priority over good spatial resolution,[†] and partly because these systems do not generally give any quantitative information (one exception is (4)); rather, they produce a picture ("dot scan" or "photoscan") of the distribution. A scanning system not limited in these ways can reduce the time, number of animals, and technical skill required to carry out distribution studies in which spatial resolution at the organ level is adequate.

These considerations led us to construct an experimental[‡] small animal scanner capable of producing a relatively high resolution photograph of the distribution of gamma emitting isotopes. In addition the system produces a profile graph of the distribution, and a measure of the total radioactivity in the animal.

DESIGN

The Argonne Cancer Research Hospital small animal scanner consists of a fixed gamma ray detector, a moving animal stage, and a photographic recording system. The animal to be scanned is anesthetized, secured on a lucite "boat" by four padded leg clamps and placed on the lucite stage which moves in a rectilinear pattern (maximum area 10 in. x 10 in.) under the detector (Figs. 1 and 2).

The scintillation detector consists of a 2 in. x 2 in. NaI (Tl) crystal and an RCA-6810 photomultiplier tube. A beryllium window on the crystal permits efficient detection of very low energy photons. The 2-in. lead detector shield is adequate for gammas up to approximately 500 KeV.

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[*] This report is taken from a paper that appears in the International Journal of Applied Radiation and Isotopes.

[†] To scan mice with adequate resolution it is necessary to reduce the collimator field of view by a factor of ~ 2 from that most commonly used for human thyroid scanning; this reduces the counting efficiency by a factor of ~ 4.

[‡] The system grew out of a laboratory setup for "phantom" scans to study methods for improving the design of scanning systems for clinical use.
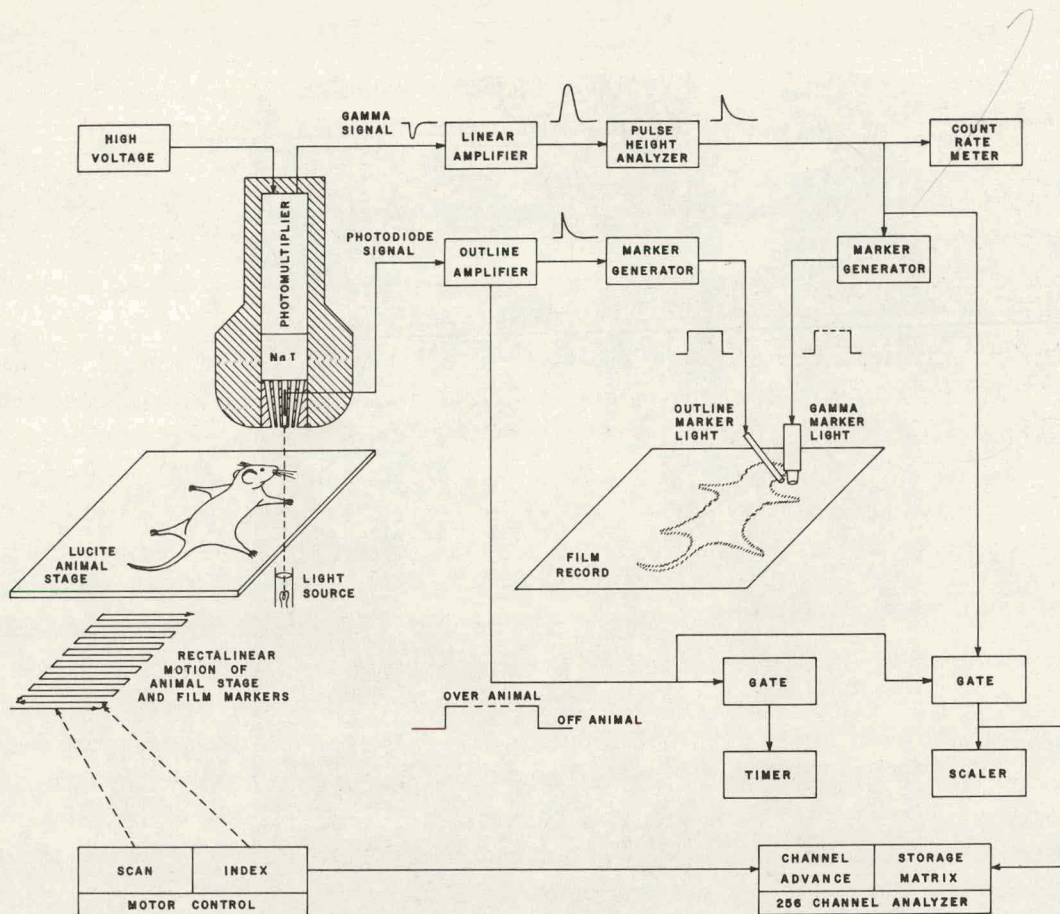
Figure 1. Functional block diagram of the small animal scanner.

Four interchangeable focused collimators having 0.25 in. and 0.50 in. diameters of view at the focus (2 in. from the collimator face) have been designed for both Hg^{203} and I^{131} radiations. Detected gammas produce pulses at the photomultiplier anode. After amplification, pulses which fall within the photopeak of the spectrum are selected by a pulse height analyzer for recording. Each selected gamma pulse produces a bell-shaped spot on film. The shape of the spot corresponds approximately to that of the collimator response function at the focal distance. These spots are produced by a pulsed light source resembling a slide projector in miniature, the "projection lamp" consisting of three Amperex 6977 triode indicator tubes. Spot intensity is controlled by selected pulse length (50, 100, 250, 500 microseconds), and spot size (0.25 in., 0.50 in. diameter) is determined by the choice of objective lens position. Thus the spot size may be selected to match the diameter of view of the particular collimator in use. As the animal is scanned, flexible coupling cables drive the light projector over the film, duplicating the animal stage motion, and producing a photograph of the distribution of detected gammas. Neglecting self absorption, this photograph is interpreted as a two-dimensional projection of the distribution of radioactivity in the animal (Fig. 3).

Concurrently with the scan, an outline (Fig. 3) of the animal is produced on the film record of activity distribution by means of an interrupted light beam and associated film marker. A light source located under the transparent animal stage is focused on a 1N2175 photodiode in the center hole of the collimator (Fig. 1). As the stage moves, the beam is broken by the animal's
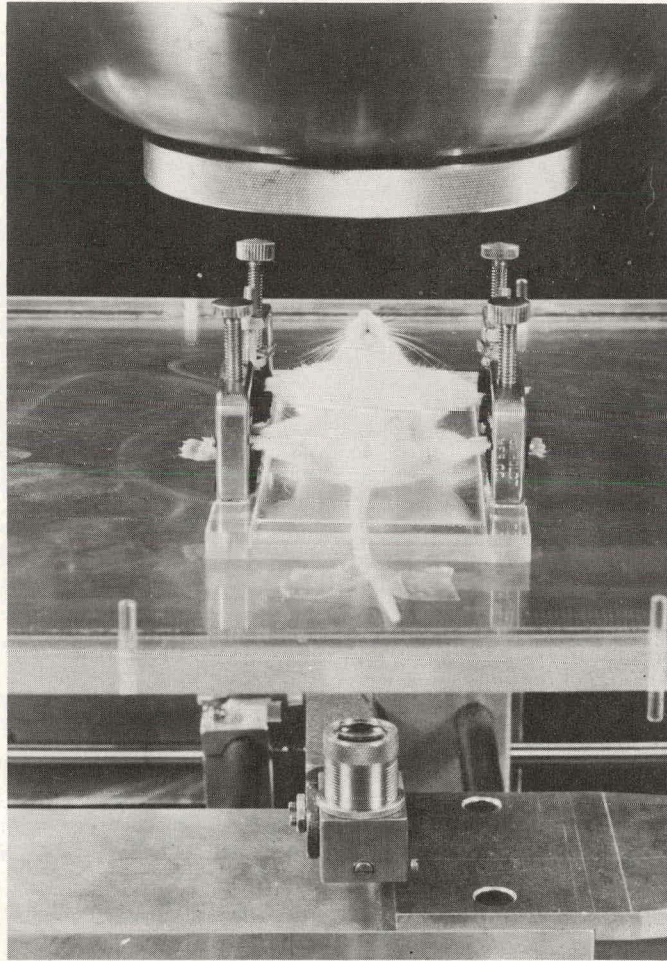
Figure 2. Anesthetized mouse held in scan position by four padded leg clamps. Fixed detector is above the animal; focused light beam is below.

body producing an intense outline mark on film. This marker light is a single Amperex 6977 focused to the same position on the film as the gamma marker light.

In addition to producing an outline of the animal, the interrupted light circuit turns on a scaler and timer when the detector is over the animal. Thus the total gamma count and time over the animal are recorded. From these data, the average count rate over the animal can be computed.

In addition, all selected gamma pulses occurring on one pass over the animal are recorded in one channel of the storage matrix of a 256 channel analyzer.[*] Signals from the index system (Fig. 1) advance the storage channel so that a "profile" graph of the activity distribution is recorded (Fig. 3). This graph is identical to that which would be produced by a "linear scanner"[2] employing a slit collimator with the same resolution.

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[*]This function could be performed by other, and simpler, count integrator-recorder systems.
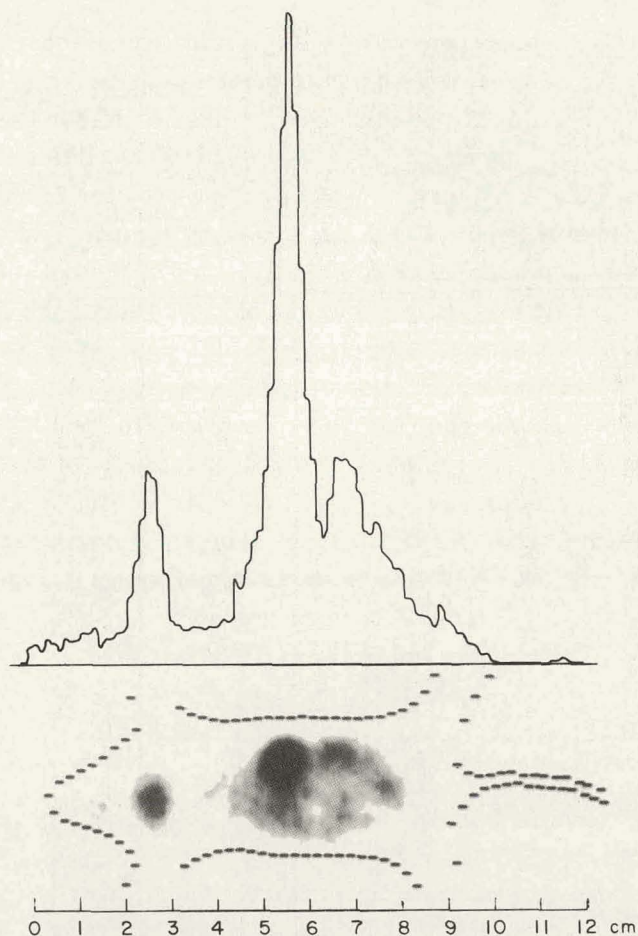
Figure 3. <u>Lower</u> - Distribution of Tc^{99m} in a 20 g mouse 4 hours after injection in tail vein. Time over the animal, 3-1/2 min. Accumulated count, 6011. Total activity ~ 30 μc. <u>Upper</u> - Profile graph of the distribution recorded on the 256 channel analyzer.

DISCUSSION

A. Resolution and Picture Quality

Ideally, scanning systems of the type discussed here produce an accurate picture of the three-dimensional distribution of radioactivity projected onto a plane. The accuracy of this picture depends on several factors, some of which are design parameters.

(1) <u>Collimator resolution and efficiency</u>. Since a relatively high radiation dose to an experimental animal can usually be tolerated, the compromise between collimator efficiency and resolution has been made in favor of resolution. Thus collimators having a maximum field of view of 0.25 in. in diameter at the focus (the diameter at half maximum response is approximately 0.125 in.) have been designed for scanning mice, and of 0.50 in. in diameter for rats (diameter at half maximum response of approximately 0.250 in.). In each case the collimator consists of an hexagonal array of round holes, tapered toward the focal point, and cast in lead. These collimators produce fairly steep gradients of count rate which outline regions of radioactivity sharply.

(2) Noise, background and septum penetration. Any detected gamma which does not originate within the collimator field of view contributes to the noise or background and tends to mask the signal due to gammas from within the field of view. Pulse height selection is used to minimize the recording of background and scattered radiation; however, this technique does nothing to eliminate photopeak pulses due to gammas which enter the detector from outside the field of view by penetrating the collimator septa. The number of such gammas can be decreased by making the septa thick, but this reduces the geometrical efficiency of the collimator. The problem of collimator design is to find that septum thickness which will reduce the penetration efficiency to some small fraction of the geometrical efficiency when the collimator looks at a large source. Since the required septum thickness is a function of gamma energy, collimators have been designed for specific isotopes to provide maximum geometrical efficiency for a selected resolution, and acceptably small penetration fraction.[5] The response field* of such a collimator, designed for Hg^{203} (279 KeV; 0.25 in. diameter of view; 0.01 penetration fraction) is shown in Figure 4. In this case the collimator consists of 127 tapered holes having 0.125 in. diameter at the crystal face, cast in lead 2 in. thick. This collimator is 65 per cent more efficient than a colli-
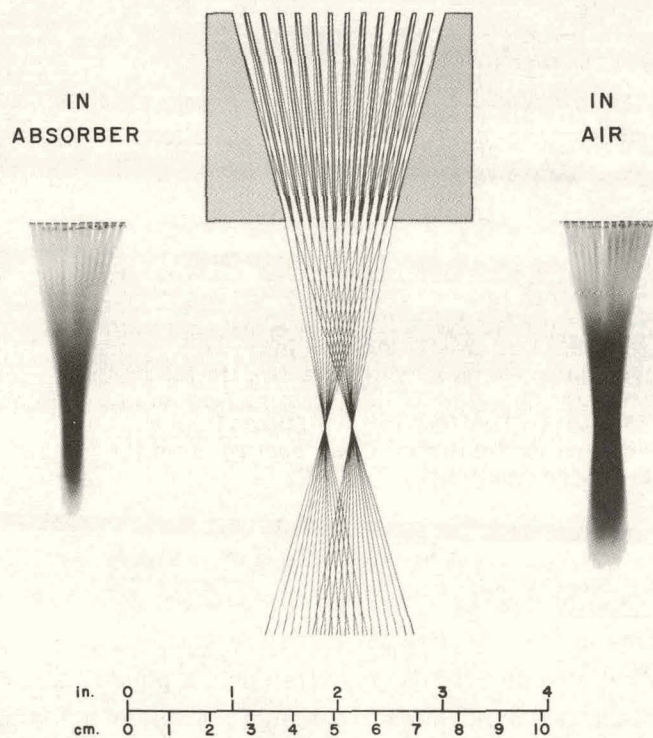

Figure 4. Center - Overlapping field of view for one plane of holes. Right - (In air). Collimator response field. Left - (In absorber). Suggests the decrease in response with depth, for activity in an absorbing medium.

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*The response field photographs were made by placing a uniform sheet distribution of I^{125} (27.4 KeV x-rays) against the crystal side of the collimator and a sheet of film on the collimator axis in what is normally the source field. Photons passing "backwards" through the collimator expose the film. By reciprocity, this interchange of source and detector (film in this case)

mator with the same resolution and penetration fraction designed for I^{131} (364 KeV) because the latter has thicker septa. Near the focal point, a cross section of the collimator response is a bell-shaped surface with a width at half height of approximately 0.125 in. (for the collimator in Fig. 4).

(3) Spot size and shape. For each selected gamma, the spot recorded on film has a bell shape which is intended to reproduce the collimator response at the focal distance. Thus the position information given by the collimator is recorded with little distortion. In addition, these spots overlap to form a more or less smooth continuous image which is a more accurate representation of the actual distribution of activity than results from recording small dots, bars, uniform discs, etc.

(4) Contrast enhancement and scanning mode. In medical scanning, small differences in count rate in adjacent areas may be clinically significant but difficult to detect by visual inspection of the scan record. This has led to the development of contrast enhancement techniques in the form of "background erase," count rate modulated spot intensity, etc. In all such techniques, the impression recorded for a detected gamma depends on the count rate which existed at the time of detection. Thus it is necessary to scan slowly enough that the count rate at each point can be determined with sufficient accuracy to set a stable threshold level or to prevent wide fluctuations in spot intensity. A slow scan speed necessitates relatively wide spacing of lines for a given area to be covered in an equivalent scanning time. This results in the coarse-raster television picture appearance which is characteristic of most medical scan records. If the scan speed is too great for the rate meter circuitry to follow changes in count rate, "scalloping" results. This has the appearance of a television picture with alternate lines displaced horizontally. While contrast is enhanced by these techniques, the accompanying line structure and scalloping tend to break up smooth contours in the distribution of activity. Studies of human perception indicate that contours which are discontinuous[6] or imbedded in a system of parallel lines[7,8] tend to be concealed.

To minimize these artifacts in the small animal scanner, no provision is made for contrast enhancement during the scan. To further reduce count rate sensitivity of the recording system, the Amperex 6977 was selected for its short decay time (approximately 5 microseconds by measurement with a photomultiplier having S11 response) and a light intensity independent of duty cycle. Under these conditions it is possible to scan the animal at high speed (without producing scalloping) making small (1/24 in.) index steps between lines. This virtually eliminates the scan line artifact.

Contrast enhancement can be regained, if desired, by viewing the film record on closed circuit television,[9] flying spot scanner[10] or by photographic manipulation, as shown in Figure 5. In addition, these (post-scan) contrast enhancement techniques eliminate the necessity of an a priori judgment as to "how much background should be erased," "how much spot intensity modulation should be introduced," etc.

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reproduces the geometrical response field provided that the film is not exposed by: a) scattered radiation, and b) photons which have penetrated the collimator septa. These conditions are approximately satisfied by I^{125} radiation, even when the film is sandwiched between two blocks of press-wood absorbing material, to simulate collimator response to a uniform distribution of activity in an absorbing medium.
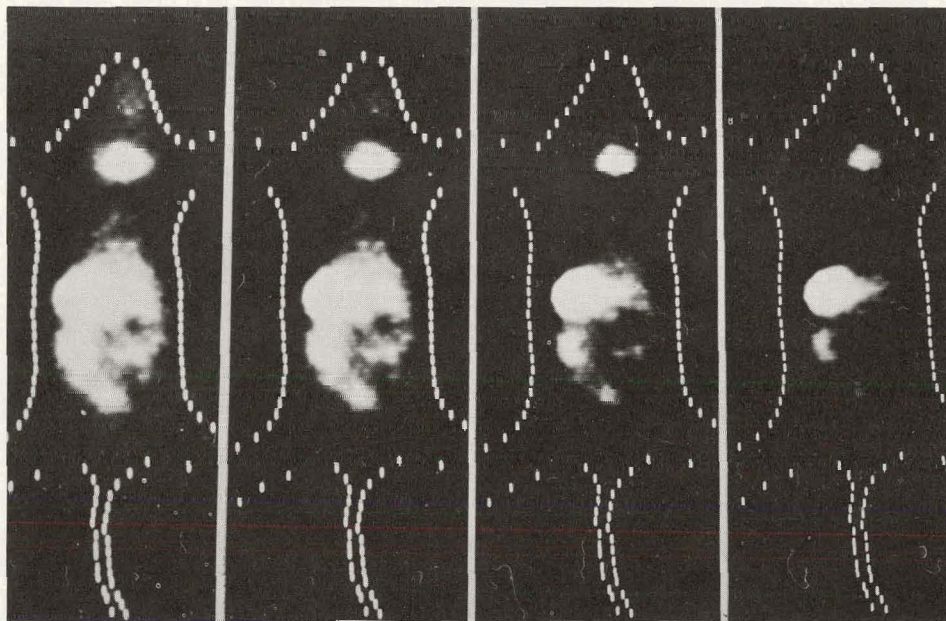
Figure 5. Four levels of "background cutoff" from a single scan
record by varying the exposure during printing.

B. Whole-Body Count

An average count rate can be computed from the total accumulated count and time over the
animal. This average rate is proportional to the total activity in the animal. For the proportion-
ality factor to be constant for successive scans, it is necessary that the following conditions be
satisfied for each scan:

(i)   The whole animal is scanned.

(ii)   The animal's body occupies the same projected area.

(iii) Changes in self-absorption with distribution can be neglected.

If these conditions are satisfied, ratios of average count rates on successive scans corre-
spond to ratios of total activity in the animal.

By using as reference the average count rate observed during a scan made immediately
after injection of a known quantity of activity, we obtain a quantitative measure of the amount of
activity in the animal on successive scans.

It should be noted that in any case, the average count rate is independent of the scan speed
used. This is important when biological half-life of the radioactive material is to be determined,
since lower scan speeds must be used as the amount of activity decreases with successive scans.

C.   Regional Distribution

The total area under the profile graph is also proportional to the total activity in the ani-
mal, but in this case, the proportionality factor is not independent of scan speed. The fraction
of the total activity in any region of the body is equal to the ratio of the corresponding area un-
der the profile graph to the total area. This fraction times the activity (as measured in B, above)
gives the quantity of activity in that region. When the activity in a region is localized in a single

organ (as determined from the photograph of the distribution) we then have a measure of the quantity of activity in that organ.

CONCLUSIONS

The scanning system provides a relatively high resolution photograph of the distribution of radioactivity in an experimental animal, together with quantitative measure of the whole-body activity and regional distribution. Since scans are made without sacrificing the animal, it is possible to study changes in the distribution and biological half-life of the isotope by repeated scans of the same animal.

While good resolution is obtained, in part, at the cost of a relatively high radiation dosage (a compromise not recommended in human scanning), other features affecting resolution and definition (such as collimators designed for specific isotopes; recording of bell-shaped spots; high speed-small step scanning mode) are applicable to medical scanning systems.

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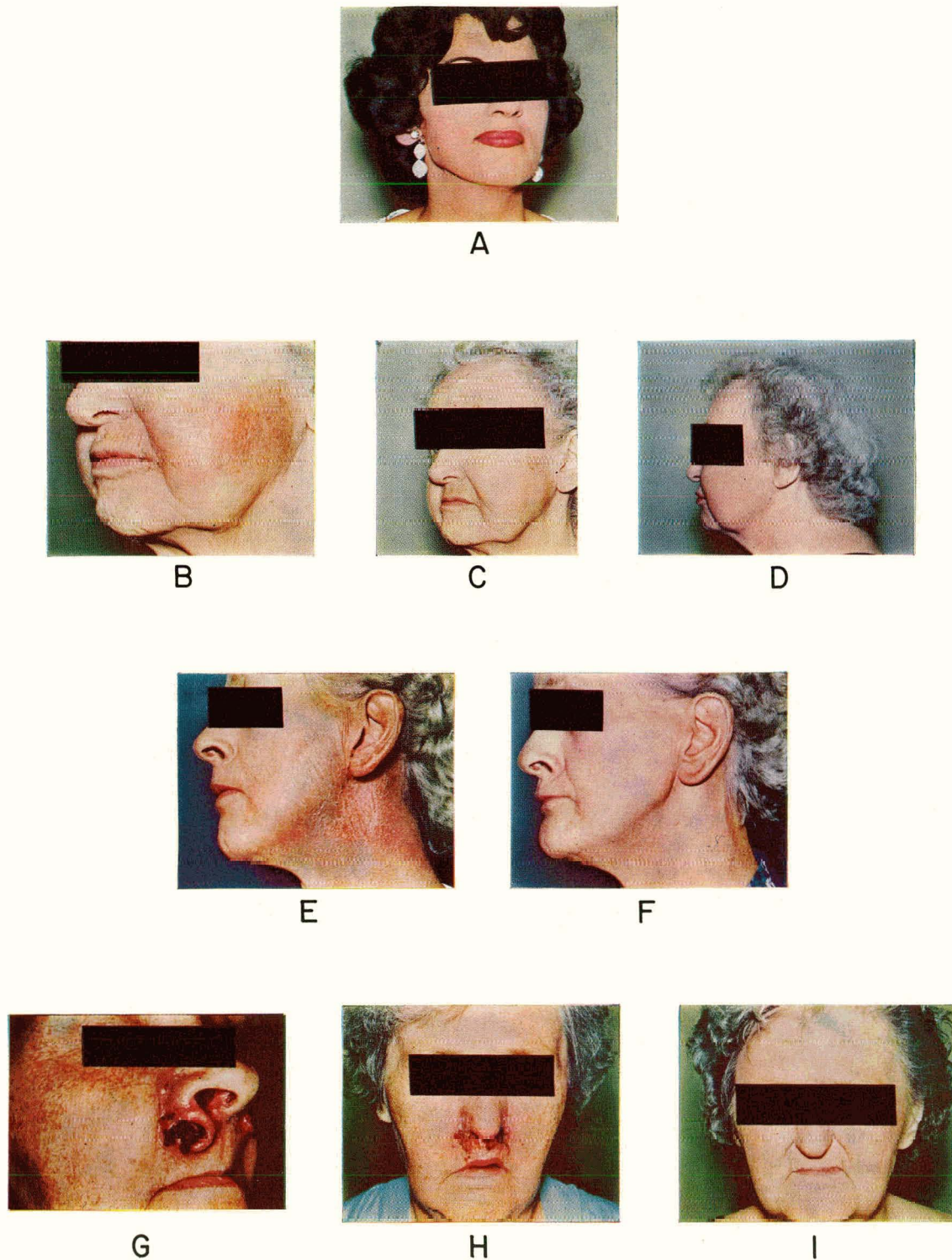
Compiled by Frances J. Skozen

FIG. 9. (*A*) Appearance of skin of face in patient (H.C.) receiving a tumor dose (5 cm. depth) of 6,000 rads 3 years previously. (*B*) Skin reaction in patient (V.S.) at maximum tissue dose of 6,800 rads in 31 treatments. (*C*) Same patient (V.S.) after $2\frac{1}{2}$ weeks. (*D*) Same patient (V.S.) after 6 months. (*E*) Skin reaction in tonsil portal in patient (N.M.). Tumor dose of 6,450 rads in 24 treatments. (*F*) Patient (N.M.) after 1 month. (*G*) Patient (A.McF.) prior to treatment. (*H*) Patient (A.McF.); wet reaction after 6,700 rads at surface of lesion in 35 treatments. (*I*) Patient (A.McF.) 9 months after treatment.