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METABOLIC CHANGES IN HUMANS FOLLOWING TOTAL-BODY IRRADIATION, Report for May 1, 1966-April 30, 1967.

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REPORT PERIOD

May 1, 1966 through April 30, 1967

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May 1, 1966 through April 30, 1967

Eugene L. Saenger, M.D.
Ben I. Friedman, M.D.
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James G. Kereiakes, Ph.D.
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PREPARING AGENCY

University of Cincinnati College of Medicine

Cincinnati General Hospital

Cincinnati, Ohio 45229

DA-49-146-XZ-315

This research has been sponsored by the Defense Atomic Support Agency NWER Subtask MC009.

FOREWORD

This report was prepared by the following members of the University of Cincinnati College of Medicine:

Eugene L. Saenger, M.D.

Ben I. Friedman, M.D.

Harry Horwitz, M.D.

James G. Kereiakes, Ph.D.

Harold Perry, M.D.

The research was supported by the Medical Division, Defense Atomic Support Agency, Washington, D.C. The Project Officer for the contract is Cdr. George P. Douglas.

In conducting the research described in this report the investigators adhered to the "Principles of Laboratory Animal Care as Established by the National Society for Medical Research."

ABSTRACT

Clinical observations in three patients treated with 100-200 rad partial body radiation to the lower body and one patient treated with 150 rad total body radiation have paralled observations previously reported.

A simple infusion filtration system for reinfusion of human bone marrow has been made. A rapid and sensitive assay method for urinary deoxycytidine which allows the detection of as little as 0.1 μg of CdR in 1 ml of urine has been developed. Two separate strains of phage to be used for antibody titration are being grown on synthetic culture media.

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INTRODUCTION:

This report presents data accumulated from May 1, 1966 through April 30, 1967. Included are clinical studies on three patients who received 100-200 rad midline absorbed tissue dose (145 to 310 R midline air exposure) of partial body radiation (PBR) to the lower body and one patient who was treated with 150 rad (226 R) total body radiation (TBR). One additional patient received "sham radiation therapy."

Major laboratory accomplishments occurred during this year. First was the completion of an infusion filtration system for reinfusion of autologous stored human bone marrow. Since this instrumentation will make infusion of marrow a safer and more easily controlled procedure we feel that earlier infusion to prevent the hematologic depression from radiation should be investigated.

Second was the perfection by Dr. I-Wen Chen of a new, much improved method for the determination of deoxycytidine (CdR) in urine from humans and from rats. This step portends future progress in understanding the metabolic pathways of nucleic acid metabolism following irradiation.

The third laboratory "break through" has been the growth of two strains of phage on synthetic culture medium in the Immunology Laboratory of Dr. Evelyn Hess. After these strains have been checked for pyrogenicity and safety, it will be

possible to titrate antibody production in experimental animals and man before and after irradiation.

An extensive report of all our studies through April 30, 1966 has been published (DASA 1844). This interim report will discuss new developments only.

Aims of the Project, Design of Study, Technique of Study, Data Collection, and Dosimetry remain essentially as reported.

Clinical Observations: Observations have been similar to those previously reported and are included in the Clinical Summaries - - Cases #066-070. Included with them are two figures (Figures 1 and 2) which graph the hematologic course of patients #66 and #70 to illustrate representative differences between responses to partial and total body radiation. The pattern after reinfusion of the bone marrow (Figure 2) may suggest repopulation of the marrow space, but the marrow was infused too late to definitely interpret the results in that way.

Enclosed with this report is a copy of a paper to be presented in Paris, France, June 26, 1967 (Attachment A - figures omitted). There is described an easy-to-use infusion filtration system for stored autologous human bone marrow. It can be sterilized and made pyrogen free. In vitro tests indicate that filtered autologous human bone marrow would effectively control the hematologic depression following radiation injury.

Biochemical Studies

The studies investigating the effects of radiation on nucleosides, especially deoxycytidine, have been limited by methodology. The enclosed report (Attachment B) covers progress on a rapid colorimetric method for determining deoxycytidine (CdR) in the urine. As little as 4×10^{-3} μmoles of deoxycytidine in 10 ml of urine can be assayed. It is separated from other 2-deoxyribose containing compounds by cation and anion exchange columns. Recovery, as evaluated using deoxycytidine-2-^{14}C, has been better than 90%.

Immunology

In the past we have attempted to study the effects of total and partial body irradiation on immunologic function through the use of C-reactive protein, thyroglobulin agglutination, latex fixation and the quantitative precipitin reaction described by Luzzio. Strains of phage were proposed as antigens which could be titrated before and after radiation. Since responses of various strains of phage are comparable, changes in titer would substantiate a radiation effect.

The phage must be grown on non-antigenic synthetic media. Until the last year the method for growing phage on synthetic culture medium was not functioning. It is possible now. After pyrogen studies have been completed on rabbits, Dr. Evelyn Hess

in the Division of Immunology is going to study titers in experimental animals for reproducibility. Titers will be compared in experimental animals before and after radiation. If all of these studies are safe, they will be carried out in humans receiving therapeutic irradiation.

Psychiatric Evaluation and Testing

The approach used has been summarized in DASA 1844. After a review of the data studied to date, there has been confirmation of the results noted previously.

Future Plans

The results in all patients treated during this project have indicated a need for further investigation of the processes studied at increasingly higher doses of total and partial body irradiation. Assessment of the value of stored human bone marrow in managing the hematological aspects of radiation injury is now feasible. Developments in the assay of deoxycytidine may ultimately help in the determination of a biological dosimeter. Alterations in antibody production and/or destruction in human beings due to radiation are going to be evaluated. These observations of the effects of radiation exposure will yield a better understanding for military planning and triage.

STUDY NO. 066

PATIENT W. H.

CHART NO. CGH - 414133

This patient was a 63 year old Negro male who was admitted to Cincinnati General Hospital February 14, 1966, with a bleeding rectal mass. Biopsy at sigmoidoscopy February 14, 1966, revealed an inoperable adenocarcinoma of the rectum with peritoneal implants and liver metastases (S.P. 66-489). A double barrel sigmoid colostomy was performed March 5, 1966. Cystoscopy showed involvement of the bladder neck.

He was re-admitted June 6, 1966 for rectal bleeding. Approximately 200 ml of bone marrow was stored without difficulty on June 7, 1966. He received sham irradiation June 9, 1966, with no adverse effects.

June 11, 1966, the patient received 200 rad midline absorbed tissue dose (310 R midline air exposure) of lower partial body radiation. He experienced no symptoms following PBR, and the hemogram remained stable.

He expired January 10, 1967, in a nursing home, 212 days post PBR.

STUDY NO. 067

PATIENT A. J.

CHART NO. CGH - 451949

This patient, a 52 year old Negro female, was admitted to Cincinnati General Hospital September 5, 1966, with nausea and left lower quadrant pain. On September 8, 1966, a barium enema revealed an obstructed sigmoid colon. A transverse colostomy and a liver biopsy were performed September 14, 1966. The biopsy report (#SP-66-2987) revealed metastatic adenocarcinoma of the liver. (Diagnosis: Carcinoma of the Sigmoid Colon with Liver Metastases).

On October 7, 1966, approximately 100 ml of bone marrow was aspirated and stored. She was shammed on October 10th, 11th, and 12th, 1966, with no adverse side effects.

The patient was treated with 100 rad midline absorbed tissue dose (145 R midline air exposure) of partial body radiation to the lower body on October 13, 1966. She experienced no ill effects from her treatment. Blood counts remained stable.

The patient was discharged October 21, 1966.

On November 2, 1966, she had nausea and vomiting and was given Compazine 10 mgms. q4h prn. Her appetite improved, and she was stronger. On March 25, 1967 (163 days post PBR), she expired.

STUDY NO. 068

PATIENT W. T.

CHART NO. CGH - 18966

This patient, a 76 year old Negro female, was admitted October 10, 1966, with left lower quadrant pain, weight loss of pproximately 17 pounds and anorexia.

In September, 1953, she had a cervical carcinoma, stage I, treated with radium and external x-ray. In May, 1954, severe post radiation proctitis necessitated loop colostomy. There were admissions in June, 1955, and June, 1956, for post radiation stenosis of the rectum, but surgery was not required. In January, 1957, post radiation stenosis of the rectum required an anterior resection of the recto-sigmoid colon with end to end anastomosis. In May, 1957, a revision of the colostomy and cecostomy was performed.

On January 7, 1965, pelvic examination revealed a midline cystic mass, inseparable from the uterus. The patient was admitted January 13, 1965, for an exploratory laparotomy, hysterectomy, and left salpingo-oophorectomy. The biopsy report (#SP 65-244) revealed endometrial papillary carcinoma.

On October 21, 1966, approximately 200 ml of bone marrow was aspirated from the left and right posterior crests and stored. She was shammed October 25, and 26, 1966, with no adverse side effects.

The patient was treated with 150 rad midline absorbed tissue dose (226 R midline air exposure) of lower partial body radiation on October 27, 1966. She experienced severe abdominal pain, diarrhea, weakness, anorexia, and confusion. On October 28, her temperature rose to 102.6° F., due to a urinary tract infection which was controlled with Gantrisin. She was discharged on November 22, 1966.

On December 1, 1966, she was admitted because of disorientation and inanition. On December 5, 1966, sternal and left anterior marrows were normocellular. Her hemogram remained stable throughout her illness with the exception of elevation of her white counts due to urinary tract infections.

The patient remained anorexic, extremely lethargic and continued a downhill course until December 22, 1966, when she expired (59 days post PBR).

STUDY NO. 069

PATIENT E, C.

CHART NO. CGH - 446-455

This patient, a 71 year old Caucasian female, was admitted to Cincinnati General Hospital February 15, 1966, with a history of "intestinal troubles" for a duration of many years. Her chief complaints at this time were anorexia without vomiting,

severe flatulence, diarrhea 1-2 x per day for the past two weeks and dull throbbing pain in the right lower quadrant.

Abdominal x-rays on February 15, 1966 revealed a huge abdominal mass. A subtotal hysterectomy and bilateral salpingo -oophorectomy was performed March 4, 1966. Biopsy report (#SP 66-702) revealed papillary cyst adenocarcinoma, right ovary. She was discharged on March 17, 1966.

The patient was treated on an out-patient basis with Cobalt 60 teletherapy 4000 R minimal tumor dose to the lower abdomen from March 15, 1966, to April 11, 1966. The upper abdomen received 4000 R minimal tumor dose from April 12, 1966, to May 20, 1966.

On December 7, 1966, the patient was readmitted with 2+ pitting edema of both legs and abdominal ascites.

Approximately 200 ml of bone marrow was aspirated and stored on December 21, 1966. The patient was shammed December 22, 1966, January 12, 1967, January 17, and 18, 1967, with no adverse side effects. Her hemogram remained stable. At this time there was little or no evidence of ascites or recurrence of tumor, so partial body radiation was not given. She was discharged January 23, 1967, and is being followed in Tumor Clinic. As of April 27, 1967, her last visit to clinic, her condition remains unchanged.

STUDY NO. 070

PATIENT F. H.

CHART NO. CGH - 198032

This patient, a 62 year old Negro male, was admitted to Cincinnati General Hospital with hemoptysis and left pleuritic pain on October 25, 1966. A chest x-ray revealed a coin lesion in the left upper lobe. On October 28, 1966, a Papanicolaou smear of the sputum was positive. A brain scan on December 9, 1966 revealed a large lesion in the right parieto-occipital area. The patient was treated with 250 KVP to the head, 3000 R minimal tumor dose to the midline from December 19, 1966 to February 1, 1967.

On February 17, 1967, approximately 170 ml of bone marrow was aspirated and stored. The patient was shammed February 28, 1967 and March 1, 1967 with no adverse side effects.

On March 2, 1967, the patient was treated with 150 rad (226 R midline air exposure) total body radiation. He experienced no ill effects from his treatment.

His WBC dropped from an average of 5,500 prior to therapy to 1,100, 35 days post TBR.

The patient was given 480 ml of bone marrow suspension intravenously without filtration on March 14, 1967 (12 days post

TBR). He tolerated the procedure well with no change in temperature, pulse, respiration, or blood pressure.

Treatment with 60 Cobalt, 3000 R to the whole head, was initiated on March 28, 1967, due to severe paresis of the left arm and moderate paresis of the left leg.

On May 8, 1967, his hemogram had returned to normal; but he had increasing mental disorientation and progressive left-sided weakness.

On May 9, 1967, 68 days post TBR, the patient expired.

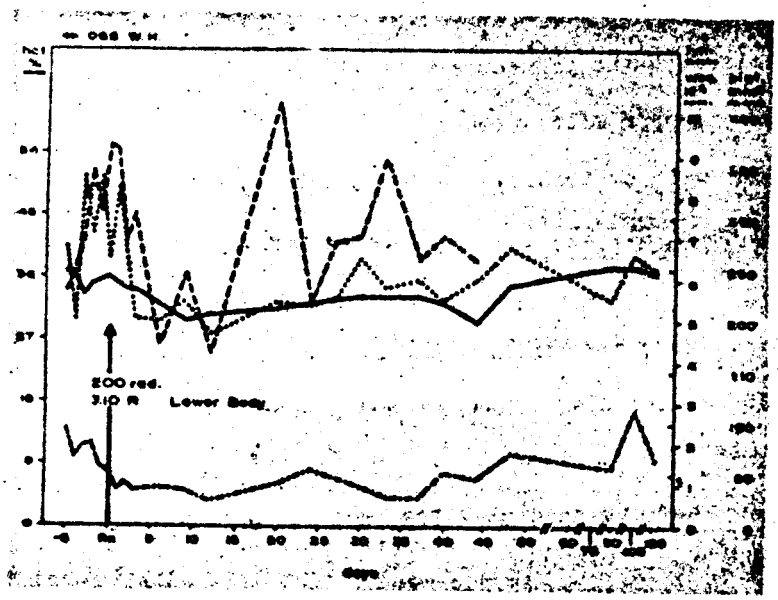

Figure 1

Figure 2

ATTACHMENT A

THE EFFECTS OF FILTRATION ON STORED HUMAN BONE MARROW

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Supported by:

Defense Atomic Support Agency DA-49-146-XZ-315

INTRODUCTION

During the last five years, we have been studying the effects on human beings of a single exposure of total body radiation which has been given for the palliation of metastatic malignancy. To date, forty patients have been so treated with 25 to 200 rad midline absorbed tissue dose (41 to 336 R midline air dose) of total body radiation. Eighteen of these patients were given 150 to 200 rad.

Marked hematologic depression occurred in all eighteen patients who received more than 125 rad total body radiation. The total elucocyte count fell below 2000 WBC/mm^3, 25 to 40 days after irradiation. The mean minimum leucocyte count of previously untreated patients who received 150 rad total body radiation alone was 1264 ± 1140 and was 1140 ± 816 when there had been previous therapy. The nadir of the leucocyte counts of patients who received 200 rad was 983 ± 366 WBC/mm^3.

Since bone marrow depression was the most life-threatening radiation effect at the doses used, we began storing bone marrow for autologous infusion after irradiation. To date, we have stored marrow from twenty five patients and have infused autologous stored human bone marrow intravenously <u>without filtration</u> into six patients. One patient had hemoglobinuria for twelve hours after infusion at 100 drops per minute. Autopsies were performed in three patients five days, eight days, and nine days respectively after reinfusion. No evidence of pulmonary emboli, pulmonary infarction, or other disease as a result of marrow infusion was found on microscopic examination.

METHODS

The bone marrow has been stored by a modification of the method previously described by Kurnick (1). Approximately 100 ml of material is aspirated from each posteior iliac crest. The marrow suspension is diluted with an equal amount of 30% glycerol -Osgood media. This mixture, in quantities of 10 ml per culture tube, is cooled at 1° Centigrade per minute to -40° Centigrade. The tubes are then placed in polyethylene bags and stored at -80° Centigrade.

On the day of infusion, the frozen marrow-glycerol-Osgood media is thawed at 4° Centigrade. Two parts of it are diluted with one part 33 1/3% dextrose in water and placed in a Fenwal plastic transfer pack. It was this mixture which was infused intravenously <u>without filtration</u> through a 17-gauge siliconized needle.

There is disagreement as to the need for filtration. Kurnick does not think it is necessary prior to infusion. Thomas, Ferrebee, and Pillow (2,3,4) recommend filtration to minimize the incidence of pulmonary embolization. Though we have not encountered embolization, we have developed a simple method of filtration for use in man.

The complete infusion filtration system consists of a Fenwal transfer pack, plastic tubing, filter assembly, and Sigmamotor pump (Figure 1). The pump has a variable speed

control, but for the experiments to be reported, has been run at 55-60 drops per minute. Within the filter assembly (Figure 2), there are successive number #50, #100 and #200 mesh filters corresponding to openings of 297, 149, and 74 microns. The filter assembly is constructed of stainless steel with Teflon washers so that it can be disassembled for cleaning. It can then be reassembled, sterilized, and made pyrogen free.

When the pump was used without a filter assembly, the plastic tubing would separate from the 17 gauge needle because of the large pieces of fibrin and marrow which would occlude the needle. With the complete system, including the filter assembly, a 25 gauge needle has been used without encountering any obstruction at the needle hub.

We have investigated the total cell count of a volume of marrow suspension and the viability of stored marrow cells before and after filtration through the infusion filtration system described. Cell counts are reported as cells per ml (1). Viability studies, as determined by dye exclusion, utilized trypan blue (5). By a modification of the method of Moorhead (6), the marrow from several patients has been cultured under phytohemagglutin stimulation for 6 days. 1.0 to 1.2 x 10^6 cells per ml are implanted in a culture media of 70% TC 199 and 30% pooled human serum (7). On the third day, the marrow is replanted, and additional phytohemagglutin is added. It is thought that the cell which differentiates and undergoes mitosis under these in vitro conditions is a hematopoietic cell.

RESULTS

We have studied bone marrow from fourteen patients. The marrow has been stored from five to twenty six months prior to study. Though the storage temperature has usually been -80° Centigrade, at times, due to transient equipment failure, the temperature has risen to as high as -40° Centigrade for as long as twenty-four hours.

Cell counts have been performed on nineteen specimens of bone marrow from fourteen patients before and after filtration (Table 1). The mean of the cell counts prior to filtration was 3.8 ± 2.0 cells x 10^6/ml and 2.0 ± 1.6 x 10^6/ml after filtration. When these means are compared by an analysis of variance, there is no significant difference in the two groups at the 5% level. The sensitivity of the cells to injury by filtration was not influenced by the duration of storage at low temperatures.

Trypan blue exclusion studies were performed on seventeen specimens of bone marrow obtained from twelve patients (Table II). The mean of the % viability before filtration was 55.3 ± 8.9% and 55.5 ± 8.1% after filtration. There was no significant difference between these two groups. Percent viability as determined by this dye exclusion technique was not influenced by the length of storage up to twenty six months nor by filtration through the system described.

Attempts to culture in vitro seven aliquots of bone marrow from six patients have yielded metaphases in two studies. In one additional study, "large mononuclear cells" with prominent

nucleoli and vacuolated cytoplasm were observed. No metaphases were seen. No growth was present in the other four cultures. Figure 3 is an example of a metaphase seen on slides from a culture of bone marrow which had been stored for 5.5 months. A metaphase from the same aliquot of bone marrow after filtration is seen in Figure 4. We have been able to culture marrow which has been stored for 13.5 months. Metaphases have been photographed from slides made from such cultures before filtration (Figure 5) and after filtration (Figures 6 and 7). A large number of metaphases has not been observed, but the presence of some is evidence of a degree of viability of the stored marrow. The number of metaphases did not decrease after filtration.

If the bone marrow has not been filtered, macroscopic clumps of material, as large as 3 mm in length, are seen passing through the tubing. On microscopic preparations from unfiltered marrow, clumping is visualized (Figures 8 and 9). Following filtration, no macroscopic particles are seen, and individual separated cells are observed on microscopic examination (Figure 10). No clumping is noted on this slide which is from the same marrow study as the two with sheets of cells.

DISCUSSION

One of the first tenets in the handling and preparation of human tissue is to minimize external manipulation whenever possible. This thought led us to the use of unfiltered marrow in the past. However, there was little question that filtered marrow would have advantages over unfiltered marrow if the ability to repopulate the marrow space was not altered by filtration. We have demonstrated that the infusion filtration system used in these experiments is easier to use than the system without the filter assembly. With the filters, a needle as small as a 25 gauge can be used for the intravenous infusion.

Since there has been no significant alteration in the cell count nor in the viability of the cells when studied in vitro, there does not appear to be a deleterious effect from filtration. Only two in vitro tests of viability were used, namely the exclusion of trypan blue dye and the capacity to grow cells in tissue culture with subsequent demonstration of mitoses. Further investigation of the cellular viability with tritium labelling of cells and acridine orange studies is advisable.

The ultimate answer as to whether filtration alters the ability of stored autologous human marrow to repopulate the marrow can come only from an adequately controlled investigation in man. This study supports the view that filtered autologous marrow should be effective in the management of myelosuppression due to radiation therapy, chemotherapy, or accidental radiation exposure in industry, in warfare, or in space.

SUMMARY

An easy-to-use infusion filtration system for autologous stored human bone marrow has been developed. There has been no difference in the means of the cell counts of marrow before or after filtration in nineteen experiments. In vivo viability studies were not changed by filtration in seventeen studies. Viability was unchanged by six to twenty six months storage at -80° Centigrade. After tissue culture for six days under phytohemagglutin stimulation, metaphases were observed in specimens obtained before and after filtration.

This study supports the concept that filtered autologous human bone marrow would be effective in controlling the severe hematologic depression associated with chemotherapy or radiation injury.

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TABLE A1

Case Number	Storage Time (months)	Cell Count (x 10^6/ml) Before Filtration	After Filtration
068	5.5	5.6	4.1
065	5.5	1.6	0.8
067	6	4.2	3.3
065	7	1.4	0.6
066	10	5.3	4.4
064	11.5	4.8	4.1
064	12	3.6	3.1
063	13.5	9.8	8.2
056	17	3.5	2.9
050	21	6.2	2.6
050	21.5	2.2	2.4
052	22	3.2	3.5
052	22.5	3.2	3.5
047	23	4.4	3.2
049	24	4.3	3.6
048	25	1.8	1.4
x1	26	3.0	2.5
x2	26	3.0	2.2
047	26	1.3	1.2
Mean ± S.D.		3.8 ± 2.0	3.0 ± 1.6

TABLE A2

Case Number	Storage Time (months)	% Viable Cells Before Filtration	% Viable Cells After Filtration
068	5.5	69	70
065	5.5	--	--
067	6	58	46
065	7	--	--
066	10	51	56
064	11.5	53	54
064	12	64	60
063	13.5	63	57
056	17	63	56
050	21	38	36
050	21.5	49	46
052	22	52	69
052	22.5	47	55
047	23	44	58
049	24	69	52
048	25	63	64
x1	26	60	52
x2	26	52	60
047	26	45	53
Mean ± S.D.		55.3 ± 8.9	55.5 ± 8.1

ATTACHMENT B

COLORIMETRIC ANALYSIS OF DEOXYCYTIDINE IN URINE AFTER SEPARATION BY ION EXCHANGE COLUMN CHROMATOGRAPHY

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Excretion of deoxycytidine (CdR) in the urine of rats exposed to ionizing radiation has been demonstrated by many investigators (1, 2, 3). In these studies, the amount of CdR excreted was based on the cysteine-sulfuric acid reaction. This reaction measures not only CdR but also many other substances containing deoxyribose (4). In a search for a suitable biological indicator of radiation dose in human beings, the urinary excretion of CdR by cancer patients treated with whole body doses of Cobalt-60 radiation was investigated in our laboratory. It was found that CdR was excreted in the post-irradiation urine, whereas no CdR could be detected either in the urine collected before irradiation or in the urine of patients with various stresses such as burn and trauma (5). The urinary excretion of CdR, therefore, may be useful as a biological indicator for the clinical evaluation of radiation injury.

The assay method for the urinary CdR involved such tedious procedures as absorption of CdR on charcoal followed by elution, two dimensional paper chromatography, and microbiological assay of the isolated CdR. The minimum detectable amount of CdR by these procedures was 0.2 μmole per 10 ml of urine. A more sensitive and rapid method for isolation and determination of urinary CdR had to be developed before the possibility of using CdR excreted in urine as a biological indicator of radiation injury could be considered. Although the spectrophotometric analysis at 280 mμ in acidic solution is a sensitive and simple method for quantitative

determination of CdR this method usually requires complete isolation of CdR from other ultraviolet-absorbing compounds. Other colorimetric methods based on the color reactions of 2-deoxyribose (dR) are not sensitive enough for our purpose (6).

The present communication deals with a sensitive colorimetric assay of CdR in the presence of other ultraviolet-absorbing substances and a rapid procedure for the isolation of CdR from the urine samples. CdR was isolated from urine by successive chromatography on columns of cation and anion exchange resins. The fraction containing CdR was used to estimate CdR content in urine by the use of the malonaldehyde-thiobarbituric acid colorimetric reaction (TBA assay). This assay was originally developed by Waravdekar and Saslaw for the estimation of deoxy sugars (7) and for the differential analysis of purine nucleosides and nucleotides (8). CdR was hydrolyzed quantitatively by heating in acidic solution and the quantities of dR released were determined by the TBA assay.

MATERIALS AND METHODS

Materials. Deoxycytidine-2-^{14}C (CdR-2-^{14}C) was obtained from Schwarz BioResearch Inc., Orangeburg, New York (10.6 μc/μmole). CdR and 2-deoxy-D-ribose (dR) were purchased from Calbiochem, Los Angeles, California. 2-Thiobarbituric acid (TBA) was a product of Eastman Organic Chemicals, Rochester, New York. It was purified by a column of Woelm acid aluminum oxide and recrystallized from warm distilled water as described by Waravdekar and Saslaw (6).

Other chemicals were all reagent grade obtained from commercial sources. AG 1-x8, 100-200 mesh (chloride form) and AG 50W-x4, 200-400 mesh (hydrogen form), were purchased from Bio-Rad Laboratories, Richmond, California. AG 1-x8 was converted to hydroxyl form by washing with 1N NaOH until the washing solution was free of chloride ions. AG 50W-x4 was washed successively with 0.5N NaOH, distilled water, and 1N HCl until absorbance at 260 mμ of each washing solution was below 0.05. Two solvent systems, isopropyl alcohol:HCl:H_2O (65:16:19, acid system) and isopropyl alcohol: concentrated ammonia:H_2O (80:10:10, alkaline system), were employed for the paper chromatographic identification of CdR and cytosine. In all cases, Whatman No. 1 papers pre-washed with 0.1N HCl were used.

Hydrolysis of CdR. Hydrolysis of CdR was performed in 2 ml of hydrochloric acid solution in a 12 ml graduated centrifuge tube. The tube, with a marble on its top, was placed in a boiling water bath. After a certain period of hydrolysis, the tube was cooled to room temperature, and the volume of hydrolyzed solution was adjusted back to 2 ml with water. For a two hour hydrolysis, the volume loss was usually about 0.1 ml. Aliquots of 0.7 ml were used to measure the amount of dR released by the use of TBA assay. In order to determine possible degradation of dR liberated from CdR during the hydrolysis, an equivalent amount of dR was also subject to the same hydrolysis conditions before the TBA assay. The optimum hydrolysis conditions with maximum hydrolysis of CdR and minimum degradation of dR were determined

by varying the hydrochloric acid concentration of the mixture and by varying hydrolysis time.

CdR-2-^{14}C was used to estimate the extent of CdR hydrolysis. 0.05 μmole of CdR-2-^{14}C (280,000 counts per minute per μmole) was hydrolyzed under various conditions as mentioned above. The solution was freeze-dried and paper chromatographed, ascending in the acid solvent system for 20 hours. This solvent system can separate cytosine from CdR. The ultraviolet-absorbing spots on the paper chromatogram corresponding to cytosine were cut into small pieces and extracted with 3 ml of 0.1N HCl in a test tube (1.5 x 15 cm) by vigorous shaking at room temperature for 2 hours. In a preliminary experiment, it was shown that recovery of cytosine from the paper chromatogram by this extraction method was better than 95%. Aliquots of the extract were counted in a liquid scintillation counter, and the amount of cytosine released was calculated from the specific activity of CdR-2-^{14}C used.

Radioactive samples were counted in a Packard Tri-Carb Liquid Scintillation Counter. A 0.5 ml sample was mixed with 20 ml of the scintillation fluid (7g PPO, 100 mg POPOP and 100g naphthalene in 1,000 ml dioxane) in a polyethylene vial for counting.

<u>TBA assay</u>. The TBA assay procedures are essentially the same as those described by Waravdekar and Saslaw (6). To 0.7 ml of sample solution was added 0.1 ml of 0.025M HIO_4 in 0.125N H_2SO_4. After 20 minutes at room temperature, 0.2 ml of 2% $NaAsO_2$ in 0.5N HCl was added, and the mixture was heated for 20 minutes in a boiling water bath with a marble on the top of the test tube.

The tube was cooled in water at room temperature for 2 minutes, and the intensity of the pink-colored solution was measured at 532 mμ, using a Beckman model DU Spectrophotometer. The amount of dR was calculated from the standard curve of dR shown in Figure 1.

Treatment of urine samples. Six hour urine specimens from two healthy persons and eight patients with various types of cancer were used for the assay. Urines from six male Wistar rats, weighing approximately 160g, were also used in this experiment.

The animals were housed in metabolism cages and fed laboratory chow and water *ad libitum*. A control urine specimen was collected during the 24 hour period prior to irradiation. The rats were placed in a lucite cage and exposed to 200 R of whole body x-radiation delivered by a Westinghouse Quadrocondex Unit operated at 250 kVcp, 15 ma, with 0.5 mm Cu and 1 mm Al added filter, 1.7 mm Cu half value layer. The dose rate was 1.4 R/min at a target-mid-animal distance of 50 cm as measured with an ionization chamber placed at center of a paraffin rat phantom. Urine specimens were collected for 24 hours following irradiation.

One twentieth of a six-hour human urine sample or a 10 ml aliquot of a twenty-four hour rat urine sample (or the total daily volume if less than 10 ml) were used for the assay. About 7,000 cpm of CdR-2-^{14}C (Specific activity: 14.7 x 10^6 cpm per μmole) were added to the urine samples as a marker for determining the loss of CdR during the isolation process. The urine samples were acidified with concentrated HCl to pH 1.0, diluted to 20 ml with water and applied on a column of 1.0 x 10 cm of AG 50W-

x4 (200-400 mesh, hydrogen form). The column was washed three times with 20 ml of water, and the absorbed CdR was eluted with 0.5M NH_4OH. CdR was always eluted out together with yellowish urinary pigments in a 5 to 10 ml fraction after 20 to 30 ml of 0.5M NH_4OH had been passed through the column. This yellowish fraction containing CdR was again applied on a column of 0.5 x 8 cm of AG 1-x8 (100-200 mesh, hydroxyl form), and the column was washed three times with 5 ml of 0.5M NH_4OH. The first 5 ml fraction from the column was discarded. All other fractions were collected in a 50 ml Erlenmeyer flask (about 20 ml) and dried in an evacuated desiccator over phosphorus pentoxide and sodium hydroxide. The residue was dissolved in 3 ml of 0.01N HCl, and insoluble substances, if any, were removed by centrifugation. pH of the supernatant fluid was usually 3 to 5 depending on urine samples and was adjusted to 2.3 $\pm$ 0.1 by careful addition of concentrated HCl. Less than 0.03 ml of concentrated HCl were needed for this pH adjustment. Aliquots from this solution were hydrolyzed in 2 ml of 5×10^{-3}N HCl (pH 2.3) for 130 minutes, and TBA assay was performed as described above. 0.5 ml aliquots from the solution were counted to determine the recovery of CdR by this isolation procedure.

RESULTS AND DISCUSSION

The colorimetric method for the determination of dR described by Waravdekar and Saslaw (7) is based on periodate oxidation and estimation of the resulting malonaldehyde with thiobarbituric acid. This method requires that the deoxypentose be in the free aldehyde form. This method was also applied for the differential colorimetric analysis of purine deoxyribonucleosides and deoxyribonucleotides (8). The hydrolysis of purine nucleosides at pH 2 for 15 minutes in a boiling water bath resulted in a quantitative liberation of dR, whereas only 37% of CdR were hydrolyzed under this condition. When the hydrolysis was carried out for 120 minutes, CdR gave rise to a maximum yield of 86%. Our results indicate that the glycosidic bonds in CdR are completely hydrolyzed at pH below 2.5 with an hydrolysis period greater than 120 minutes (Figures 2 and 3). Experiments summarized in Figure 2 show that more than 98% of cytosine was liberated from CdR at pH below 2.5. At pH 3.0, 95% of cytosine was released, whereas only 62% of CdR was hydrolyzed at pH 3.5. If the hydrolysis of CdR was followed by measuring the amount of dR released by the TBA assay, maximum hydrolysis of CdR was found in the pH range of 2.1-2.5. At pH above 2.5, the amount of dR present in the hydrolysis mixture decreased, and at pH 3.5 it represented only 54% of total CdR used. This decrease is due to an incomplete hydrolysis of CdR at this pH range, as can be seen from the decreased amount of cytosine present in the hydrolysis mixture. At pH below 2.1, the amount of dR decreased rapidly with decreasing pH, while the amount of cytosine released at this pH range showed complete

hydrolysis of CdR. These results suggest that at the lower pH range, dR released from CdR is rapidly degraded to products which do not give color in the TBA assay. Similar results are obtained when the same amounts of dR are heated under the same conditions. It is rapidly degraded at pH below 2.3, and degradation is less than 6% at pH above 2.5.

Next, the degradation of dR and the hydrolysis of CdR in 5×10^{-3}N HCl (pH 2.3) were studied with various heating times. The results are summarized in Figure 3. Hydrolysis of CdR was complete after 120 minutes heating, as can be seen from the complete conversion of CdR to cytosine. The amount of dR released from CdR reached the maximum (80% of total dR present in CdR) after 120 minutes hydrolysis and stayed at this level for at least another hour of heating. When dR was heated under these conditions, it was slowly degraded in the first hour, and the degradation reached an almost constant level (about 14%) after two hours. It is seen from these results that the optimum pH and hydrolysis period for maximum hydrolysis of CdR with minimum degradation of dR are pH 2.1 to pH 2.5 and 120 to 180 minutes. Subsequently, hydrolysis of CdR for the TBA assay was routinely carried out in 2 ml of 5×10^{-3}N HCl (pH 2.3) in a boiling water bath for 130 minutes.

Figure 1 also shows a linear relationship between CdR concentration and optical density at 532 mμ which represents the amount of dR released from CdR. Each point represents an average of five assays and the reproducibility of this assay method was found to be better than ± 3%. This linear relationship could be observed even though as much as 0.15 μmole of CdR was used for the hydrolysis.

As mentioned earlier, although CdR was hydrolyzed completely, the amount of dR assayed at the end of the hydrolysis could only account for 80% of total CdR used, presumably due to the degradation of parts of dR molecules under these hydrolysis conditions. Degradation was always 20% regardless of the amount of CdR used, up to 0.15 μmole. Therefore, it is understandable that the slope of the standard curve of CdR is only 80% of that of dR without prior heat treatment. It can be concluded, therefore, that hydrolysis of CdR in 5×10^{-3}N HCl at 100°C for 130 minutes followed by the TBA assay may be employed for the quantitative estimation of CdR. Grossman and Greenlees have taken advantage of the fact that hydrogenation of the pyrimidine ring could acid-labilize the N-deoxyribosyl linkage of pyrimidine nucleosides (9). They have reported on colorimetric analysis of catalytically reduced pyrimidine deoxyribonucleosides with the use of the malonaldehyde-thiobarbituric acid reaction (10). This method seems to require a strictly controlled hydrogenation reaction. Over-exposure to hydrogen gas could result in the rapid loss of dR due probably to the further reduction of dR to 2-deoxyribitol.

In order to estimate CdR content in urine with the use of the TBA assay, it is necessary to remove from the urine sample dR and other dR containing compounds. This was achieved easily by the use of cation and anion exchange columns as described before. Of all the deoxyribonucleosides tested, thymidine and deoxyuridine were not retained on the cation exchange column. dR and other neutral compounds such as urea were also washed out from the column with water. CdR was then eluted out, together

with yellowish urinary pigments, by 0.5 M NH_4OH. The fraction containing CdR could conveniently be identified by its dark yellow color. In fact, a yellow band migrating down the column could be seen clearly during the elution process, thus eliminating the tedious fractionation procedure usually involved in the column chromatography. All CdR was eluted in a fraction of less than 10 ml. The cation exchange column failed to separate CdR from deoxyadenosine, deoxyguanosine, and deoxyinosine. However, these purine deoxyribonucleosides were absorbed on the anion exchange column, while CdR was washed out by 0.5 M NH_4OH. Overall recovery of CdR was about 93% based on the amount of added CdR-2-^{14}C recovered at the end of the purification. These results are summarized in Table 1 and 2.

Table 1 shows the results of CdR assay on urines obtained from two healthy persons and eight patients with various types of cancer. The absorbances at 532 mμ of the partially purified CdR fractions indicate that no appreciable amount of CdR was detected in any sample within the sensitivity of this assay method. The minimum detectable amount of CdR by this assay method was calculated to be 4×10^{-3} μmole per 10 ml of urine with a reproducibility of $\pm$ 3% (Figure 1). The minimum absorbance of samples for this accuracy was found to be 0.05. This assay method is 50 times more sensitive than the method used previously (5). The absorbances at 280 mμ of the fractions show the presence of other ultraviolet-absorbing substances the amount of which varies widely from sample to sample. Paper chromatograms of these fractions in both acid and alkaline systems revealed diffused ultraviolet-

absorbing spots near CdR area. No attempt was made to identify these compounds. The possible interference of TBA assay by the impurities present in the partially purified CdR fraction was investigated by the use of an internal standard. The absorbances at 532 mμ with added CdR indicate that these impurities do not interfere with the color reaction. The absorbance at 532 mμ of 0.05 μmole of CdR in 2 ml hydrolysis solution is 0.640 (Figure 1).

This assay technique was also used on rat urine samples to see whether urinary excretion of CdR following x-radiation could be demonstrated. The results of these studies are summarized in Table 2. In contrast with the human urine, amounts of CdR were found in the rat urines collected during 24 hour period before irradiation. The average value for six experiments was 2.98 μmoles. This value was increased to about 17.3 μmoles after irradiation with 200 R. Other investigators reported values of about 5.3 and 24 μmoles, respectively (1).

In order to determine whether the pink chromogens with an absorption maximum at about 532 mμ formed during the TBA assay of the partially purified CdR fractions were indeed contributed entirely by CdR, aliquots from the partially purified CdR fractions were paper chromatographed in both acid and alkaline systems. The ultraviolet-absorbing area corresponding to CdR was quantitatively eluted and the TBA assay was performed. It was found that all the TBA-positive substance was recovered from the area corresponding to CdR. Rotherham and Schneider reported that in addition to CdR, 5-methyldeoxycytidine was also excreted by normal rats (11). The purification procedure employed in our experiment did not allow CdR

to be separated from 5-methyldeoxycytidine. 5-methyldeoxycytidine also was found to give a positive TBA assay with approximately the same molar extinction coefficient as CdR. However, we failed to detect any ultraviolet-absorbing spots corresponding to either 5-methyldeoxycytidine or 5-methylcytosine on paper chromatograms whe the partially purified CdR fraction was chromatographed in the acid solvent system either directly or after being hydrolyzed with acid.

SUMMARY

A rapid and sensitive colorimetric determination of deoxycytidine in urine has been described which can detect as little as 4×10^{-3} μmole of deoxycytidine in 10 ml of urine. Deoxycytidine in urine is separated from other 2-deoxyribose containing compounds by successive treatment with cation and anion exchange columns. Deoxycytidine-2-^{14}C is added to each sample to estimate the loss of endogenous deoxycytidine during the purification process. Recovery of deoxycytidine is better than 90%. The partially purified fraction containing deoxycytidine is hydrolyzed in 5×10^{-3}N HCl in a boiling water bath for 130 minutes, and the amount of 2-deoxyribose released is measured with the use of the malonaldehyde-thiobarbituric acid reaction. Under these conditions deoxycytidine is hydrolyzed completely, but 20% of the 2-deoxyribose released is degraded.

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TABLE B: QUANTITATIVE ASSAY OF CdR IN HUMAN URINES

A twentieth of 6-hour urine collection (maximum of 20 ml) with added CdR-2-^{14}C* was passed through ion exchange columns as described before in the text. Absorbance at 280 mμ of the partially purified CdR fraction (3 ml) was measured. A 0.5 ml aliquot from this CdR fraction was counted, and another two 1 ml aliquots were used to determine CdR content as described in the text, one of which contained 0.05 μmoles of added CdR to serve as an internal standard. Recovery of CdR was calculated from the radioactivity of CdR-2-^{14}C added to the urine and the radioactivity recovered at the end of the purification. Abbreviations used in this table are: M - male; F - female; Ca. - carcinoma; A280 or 532 - absorbance at 280 or 532 mμ.

Patients	A280	A532	A532 with added CdR	Recovery of CdR
				%
1. M., healthy	1.28	0	0.650	99
2. F., healthy	0.495	0	0.638	95
3. F., Ca. breast	0.532	0.005	0.642	90
4. F., Ca. ovaries	1.61	0	0.625	96
5. M., Ca. lymph node	0.222	0.015	0.648	91
6. M., Ca. rectum, bladder	1.06	0.007	0.644	91
7. M., Ca. small bowel	0.486	0.010	0.658	93
8. F., Ca. breast	1.03	0.005	0.635	97
9. M., Ca. colon	0.232	0.006	0.645	92
10. M., Ca. lung	2.60	0	0.640	87

* The amount of CdR-2-^{14}C added was less than 5×10^{-4} μmole (7,000 cpm) and was negligible in the TBA assay.

TABLE B2 CdR IN RAT URINES BEFORE AND AFTER X-IRRADIATION (200R)

A 10 ml aliquot of each urine sample (or the total daily volume if less than 10 ml) was used for the determination of CdR as described in Table 1. The μmoles of CdR in 24 hour urine have been corrected for the loss during the purification.

	Before Irradiation		After Irradiation	
Sample number	Recovery of CdR	CdR in 24 hour urine	Recovery of CdR	CdR in 24 hour urine
	(%)	(μmoles)	(%)	(μmoles)
1	91	3.83	98	21.0
2	93	2.36	94	20.2
3	95	3.36	84	17.3
4	92	4.32	96	21.8
5	96	1.35	95	12.9
6	94	2.68	97	10.4

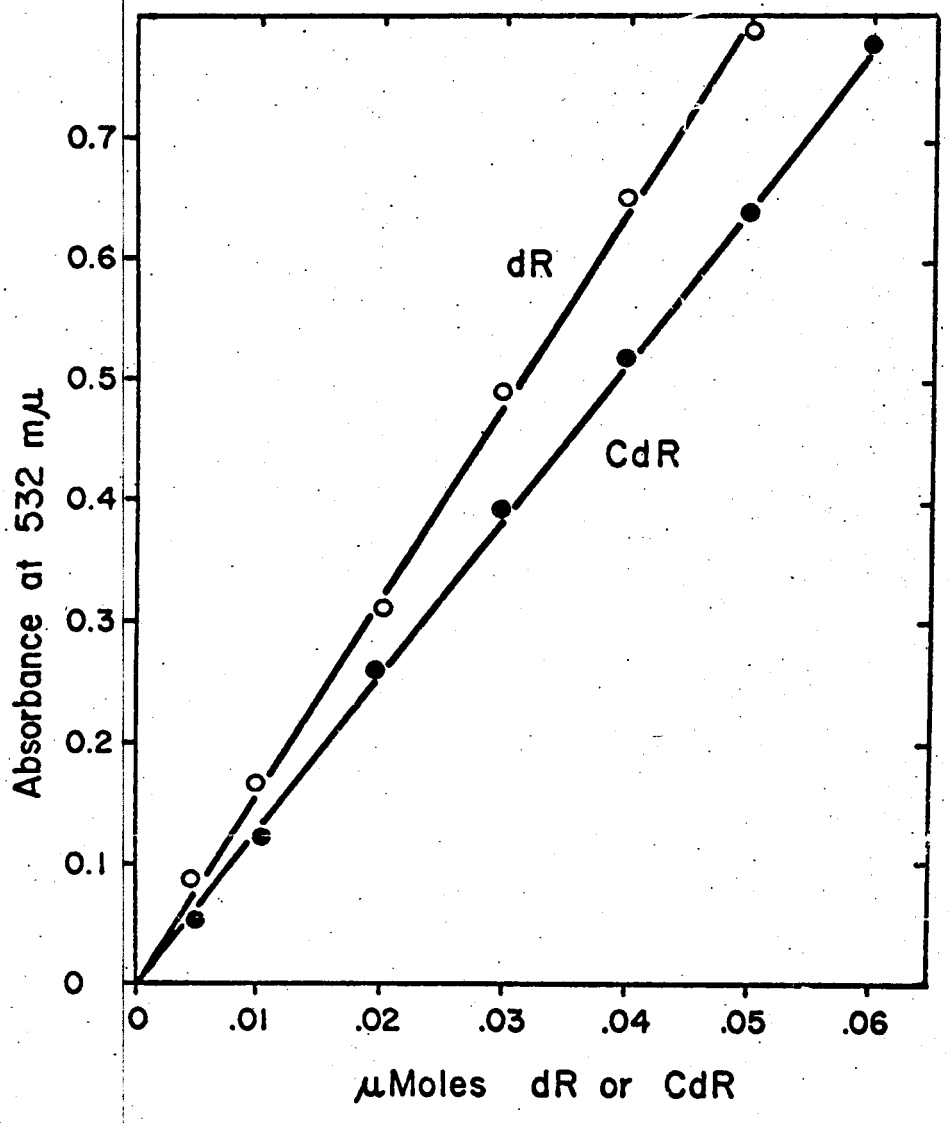

Figure B1 Relationship between concentration of dR or CdR and absorbance at 532 mμ. The abscissa represents the μmoles of dR or CdR present in 2 ml of 5 x 10^{-3}N HCl solution. The tubes containing CdR were heated in a boiling water bath for 130 minutes prior to the TBA assay. dR was assayed without prior heat treatment. The TBA assay was performed as described in the text.

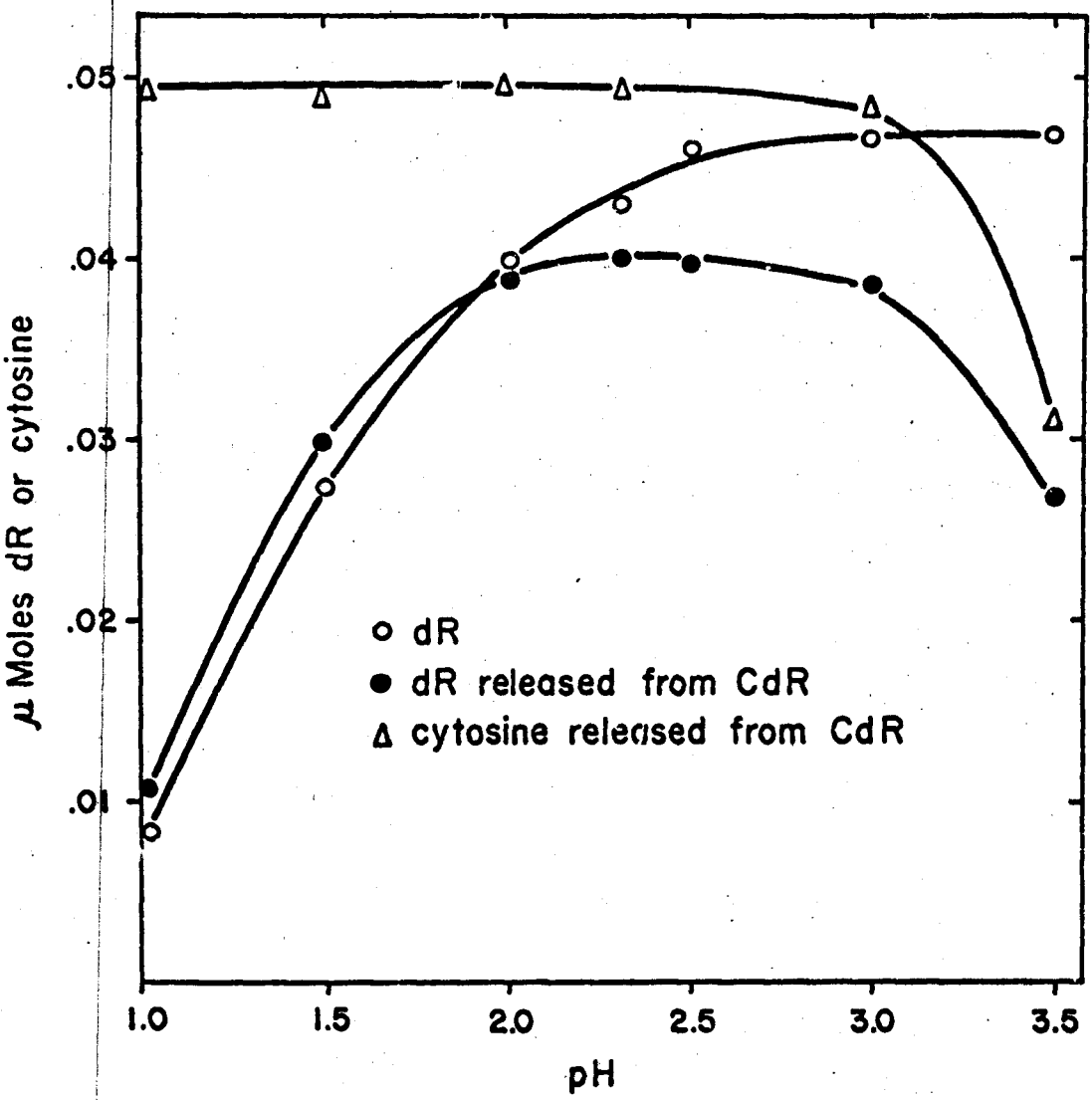

Figure B2 Effect of varying pH on the hydrolysis of CdR. 0.05 μmole each of CdR or dR was heated in 2 ml of HCl solutions with different pHs in a boiling water bath for 130 minutes. Amounts of dR and cytosine present at the end of the hydrolysis were measured as described in the text.

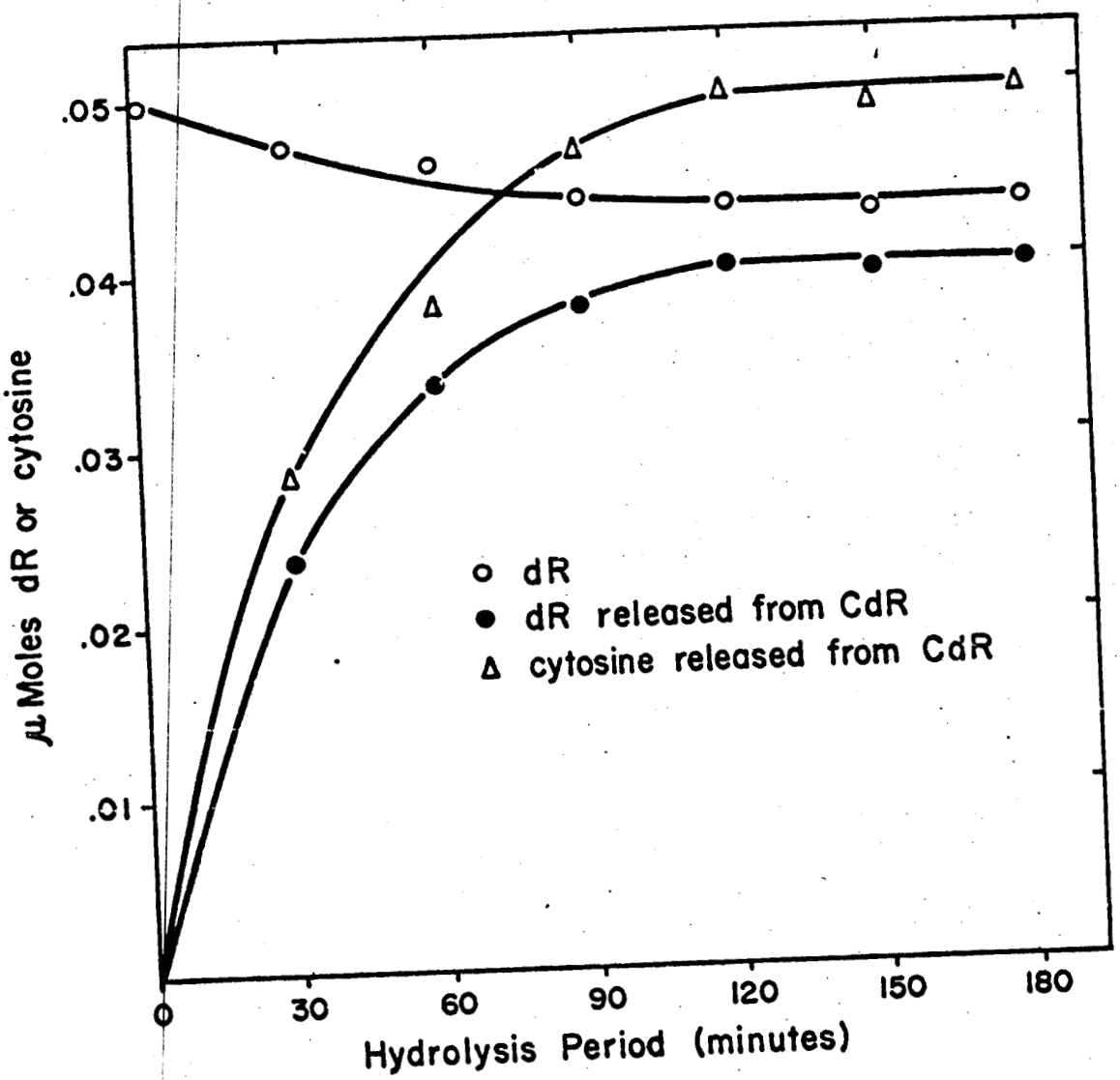

Figure B3 Effect of heating times on the hydrolysis of CdR. 0.05 μmole each of CdR or dR was heated in 2 ml of 5×10^{-3}N HCl (pH 2.3) in a boiling water bath for various periods of time. Amounts of dR and cytosine present at the end of the hydrolysis were measured as described in the text.

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13. ABSTRACT

Clinical observations in three patients treated with 100-200 rad partial body radiation to the lower body and one patient treated with 150 rad total body radiation have paralled observations previously reported.

A simple infusion filtration system for reinfusion of human bone marrow has been made. A rapid and sensitive assay method for urinary deoxycytidine which allows the detection of as little as 0.1 μg of CdR in 1 ml of urine has been developed. Two separate strains of phage to be used for antibody titration are being grown on synthetic culture media.

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