

Conjugation of Radiolabeled Polyamines in the Rat¹

Michael G. Rosenblum and Diane H. Russell²

Department of Pharmacology, University of Arizona Medical Center, Tucson, Arizona 85724

SUMMARY

Initial work reporting elevated polyamine levels in body fluids of cancer patients indicated that a percentage of the polyamine pools was present in conjugated form making hydrolysis necessary for assessment of the total polyamine content in urine and serum. In this paper, we report plasma decay curves for [¹⁴C]polyamines after i.v. administration and the temporal appearance of conjugates. Following the administration of [¹⁴C]polyamines, the radiolabel rapidly disappeared from the plasma in the order: spermidine > putrescine > spermine. Separation of the [¹⁴C]polyamines from conjugated radiolabeled compounds with Dowex chromatography indicated that [¹⁴C]putrescine and [¹⁴C]spermidine were rapidly conjugated, whereas no significant conjugation of spermine was detectable. After near-total hepatectomy of rats, there was no detectable formation of conjugates, whereas unilateral nephrectomy had little effect on the appearance of conjugates. This suggests that conjugation may take place in the liver. Free putrescine or spermidine could be regenerated from the conjugates by acid hydrolysis, suggesting that the conjugation process does not involve any alteration of the polyamines.

INTRODUCTION

Polyamine levels in the urine and/or serum of cancer patients show potential usefulness in the diagnosis of abnormal pathological states and in the assessment of response to therapy. An initial report (6) indicated that urinary polyamine levels were elevated in diagnosed cancer patients, and several other studies have reported similar elevations (10, 14). Dreyfuss *et al.* (3) found that 37 of 42 patients (88%) had measurable elevations in 1 or more polyamines in 24-hr urine specimens. Other studies have found elevations of polyamines in the urine of cancer patients in 70 to 90% of the cases (4, 5).

In a model proposed by Russell *et al.* (8, 9, 11, 12), elevated urine and plasma polyamine levels originate from intracellular pools that are released into the plasma upon cell lysis. In neoplastic disease, intracellular levels of polyamines and substantial spontaneous cell loss factors could account for the subsequent elevated urinary polyamine levels detected in cancer patients (9).

A decreased amount of spermidine in both liver and the

tumor of rats with regressing MTW9 mammary carcinomas paralleled an elevation of spermidine in the serum (9). The period of maximal tumor regression (*i.e.*, within 48 hr of removal of hormonal support) corresponded with the time of the highest level of spermidine in the serum or in the tumor interstitial fluid. This animal model suggested that intracellular spermidine levels that increase during tumor growth were lowered by excretion during regression and, further, that spermidine levels in the serum or urine reflected tumor cell death.

This concept was further supported by studies of the effects of chemotherapy or radiation therapy on polyamine levels in a rapidly growing rat hepatoma (3924A). Rapid increases in serum levels of putrescine and spermidine corresponded to decreased tumor cellularity as measured by histological techniques (11, 12). Since irradiation was confined to the tumor with no detectable involvement of the host tissue, it was concluded that the increases in putrescine and spermidine detected in the serum were derived from the tumor tissue.

Serial determinations of extracellular polyamine levels obtained from patients with hematological cancers and solid tumors before and after the initiation of cancer chemotherapy showed that a greater than 2-fold rise in spermidine was highly correlated with response to treatment (8). Further, base-line putrescine levels were found to be significantly higher in patients with active disease. The authors of this study proposed a model of spermidine as a marker of tumor cell kill and putrescine as a marker of cells progressing the cell cycle, and they suggested that polyamines in extracellular fluids may best indicate tumor kinetics rather than tumor burden. This would be compatible with data showing that slow-growing tumors have lower concentrations of putrescine and higher concentrations of spermidine, whereas rapidly growing tumors generally exhibit high concentrations of both putrescine and spermidine (7).

Early measurements of polyamines in the body fluids of humans indicated that the free levels of polyamines were exceedingly low (16). Only after plasma or urine samples were subjected to extensive alkaline or acidic hydrolysis at elevated temperatures could polyamine levels be detected by standard analytical procedures (1, 6). Russell *et al.* (10) and others (1) have suggested that polyamines found in physiological fluids are conjugated to a major extent. The nature of these conjugates as well as the possible site(s) of conjugation have not been elucidated. Clarification of polyamine-conjugative pathways and identification of polyamine-specific conjugates in the urine and plasma of cancer patients may ultimately lead to the development of rapid and specific immunological assays for polyamine conjugates in

¹ This work was supported by USPHS Grants CA-14783 and CA-17094 from the National Cancer Institute and by American Cancer Society Grant IN-110.

² Recipient of a Research Career Development Award CA-00072 from the National Cancer Institute.

Received June 28, 1976; accepted September 24, 1976.

body fluids. These assays could be valuable in rapid assessment of pathological conditions and of response to treatment.

In this paper, we report studies of plasma clearance of [^{14}C]polyamines and conjugation patterns of putrescine, spermidine, and spermine. These data suggest that conjugation occurs in the liver, since totally hepatectomized rats do not exhibit the rapid conjugation of these amines. Unilateral nephrectomy, however, does not significantly change the conjugation patterns.

MATERIALS AND METHODS

Materials. [^{14}C]Spermidine trihydrochloride (12.5 mCi/mole), [^{14}C]putrescine dihydrochloride (20.8 mCi/mole), and [^{14}C]spermine tetrahydrochloride (12.5 mCi/mole) were obtained from New England Nuclear, Boston, Mass., and used without further purification. All radiolabeled compounds contained ^{14}C in the 4-carbon chain. Putrescine, spermidine, and spermine hydrochlorides were obtained from Calbiochem, Los Angeles, Calif., and recrystallized 3 times from ethanol before use. Dowex 50W-X8 and Bio-Rex 70 were obtained from Bio-Rad Laboratories, Richmond, Calif.

Determination of the Disappearance of [^{14}C]Polyamines from Plasma Samples of Rats. Male Sprague-Dawley rats (350 to 400 g) were anesthetized with sodium thiopental (25 mg i.p.). Cannulas were placed in the right caudal artery and the left caudal vein. At 20 min after this surgical procedure, 2.5 μCi of [^{14}C]spermidine, [^{14}C]spermine, or [^{14}C]putrescine were injected i.v. in 0.5 ml of 0.9% NaCl solution. This amount of label corresponds to 20 nmoles of spermine and spermidine and 12 nmoles of putrescine. Arterial blood samples (0.5 ml) were obtained 1, 2, 4, 5, 10, 15, 20, 30, and 60 min after injection. A 1-ml blood sample was obtained at 30 and 60 min after injection. After each arterial blood withdrawal, 0.5 ml of 0.9% NaCl solution was injected via the venous catheter. Each sample was placed in a 2-ml centrifuge tube and spun for 1.5 min in a Sorvall microfuge. Plasma was decanted, and a 0.1-ml aliquot was counted to determine radiolabeled ^{14}C . For determination of plasma conjugation patterns, a 0.1-ml sample of plasma was added to a centrifuge tube containing 0.1 ml cold 5% trichloroacetic acid and centrifuged for 2 min. For all plasma samples, sufficient volume was concentrated to obtain a minimum of 3000 cpm. The supernatant was neutralized with NaOH and chromatographed on Dowex as described below.

Hepatectomy and Nephrectomy. Male Sprague-Dawley rats (300 to 400 g) were subjected to subtotal hepatectomy (90%) or unilateral nephrectomy. Plasma conjugation patterns were measured in samples obtained as above immediately after surgery.

Dowex Chromatography of Urinary [^{14}C]Polyamine Conjugates. Male Sprague-Dawley rats (250 to 300 g) were given i.p. injections of 3 μCi of [^{14}C]polyamines. Each animal was then kept in an Econo metabolic cage (Scientific Products, McGraw Park, Ill.) in order to collect urine. Urine samples were collected at 4-hr intervals and immediately frozen. To obtain polyamine conjugates, urine samples

were thawed, filtered, and flash-evaporated. The solid residue was then redissolved in a minimal amount of deionized H_2O and chromatographed on Dowex 50.

Dowex 50W-X8- H^+ (200 to 400 mesh) or Bio-Rex 70- H^+ (100 to 200 mesh) was prepared by washing 50 g of the resin with 100 ml of 0.1 M HCl. The HCl was then decanted, and the resin was washed with 200 ml of 0.05 M sodium-potassium phosphate buffer or until a neutral pH was obtained. The resin was poured into a glass column (1.7 \times 20 cm). All Dowex columns were run at room temperature and eluted with a 2 to 5 M linear NaCl gradient in 0.05 M sodium-potassium phosphate buffer adjusted to pH 7.2. Bio-Rex 70 columns were also run at room temperature and eluted with 4 M NaCl in 0.05 M sodium-potassium buffer adjusted to pH 7.2. The eluates were fractionated into eighty 1-ml aliquots using a Gilson fraction collector. Alternate fractions were assayed for ^{14}C by solubilizing 0.2 ml of each fraction in 14 ml of Aquasol (New England Nuclear) and 1 ml of distilled H_2O .

Hydrolysis. For regeneration of polyamines from the conjugates, 1 ml of combined, concentrated Dowex eluants containing the conjugates was placed in a 10-ml screw-capped centrifuge tube containing 1 ml of 12 N HCl. The solution was then aerated with dry nitrogen for 5 min and sealed. The tube was heated at 110° for 14 to 16 hr. The sample was cooled, evaporated to dryness in a vacuum, and reconstituted to original volume with H_2O . An 80- μl sample of the hydrolyzed conjugate was neutralized with 6 N NaOH and chromatographed on Dowex as described above.

RESULTS AND DISCUSSION

Plasma Polyamine Levels and Polyamine Conjugates.

The plasma decay curve for ^{14}C -radiolabeled polyamines is shown in Chart 1. Within 10 min of injection, plasma [^{14}C]spermidine levels had declined by 89% and [^{14}C]putrescine levels had declined by 67% from the 1-min plasma levels indicating extensive distribution of these labels to extravascular sites. [^{14}C]Spermine was distributed only to a small extent (30%).

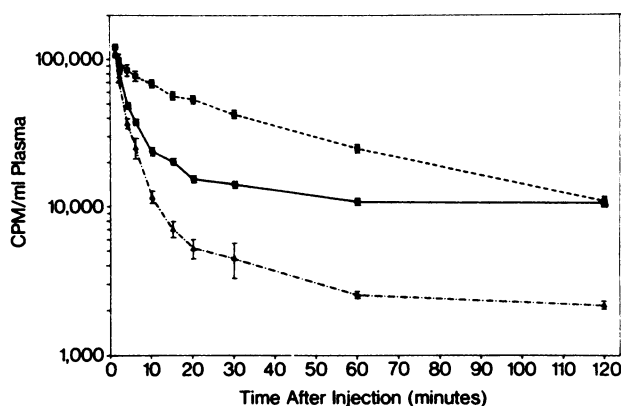


Chart 1. Disappearance of [^{14}C]polyamines from circulating plasma in the anesthetized rat. Male rats (350 to 400 g) were given i.v. injections of 2.5 μCi of spermine (\blacksquare), putrescine (\bullet), or spermidine (\blacktriangle) after light sodium thiopental anesthesia. A 0.5-ml blood sample was obtained at various times after injection, and ^{14}C activity was assayed on a 0.1-ml plasma sample in 10 ml Aquasol. Data shown are the mean \pm S.E. of 2 determinations of each time point from 3 animals for each polyamine.

The temporal appearance of [^{14}C]spermidine conjugate is shown in Chart 2. Dowex chromatograms of plasma after [^{14}C]spermidine injection showed a labeled fraction which eluted with 2.5 M NaCl. This peak was distinct from authentic spermidine which eluted in Fractions 35 to 50 with 3.5 to 4.5 M NaCl. Charts 2B (30 min after injection) and 2C (60 min after injection) indicate that the spermidine conjugate comprises a minimum of 50% of the circulating plasma label. In contrast to spermidine conjugation, putrescine conjugation (Chart 3) was more rapid. The [^{14}C]putrescine conjugate comprised 60% of the circulating label within 30 min of injection (Chart 3B). At 60 min after injection, the conjugate accounted for 90% of the ^{14}C in the plasma (Chart 3C). In contrast to the extensive conjugation of both putrescine and spermidine, there was essentially no conjugation of spermine (Chart 4).

The rapid fall in the plasma decay curve for [^{14}C]spermidine indicates that both the ^{14}C -labeled conjugate and free ^{14}C label may be distributed to extravascular sites; however, only conjugated [^{14}C]spermidine has been detected in the urine, suggesting that conjugation is neces-

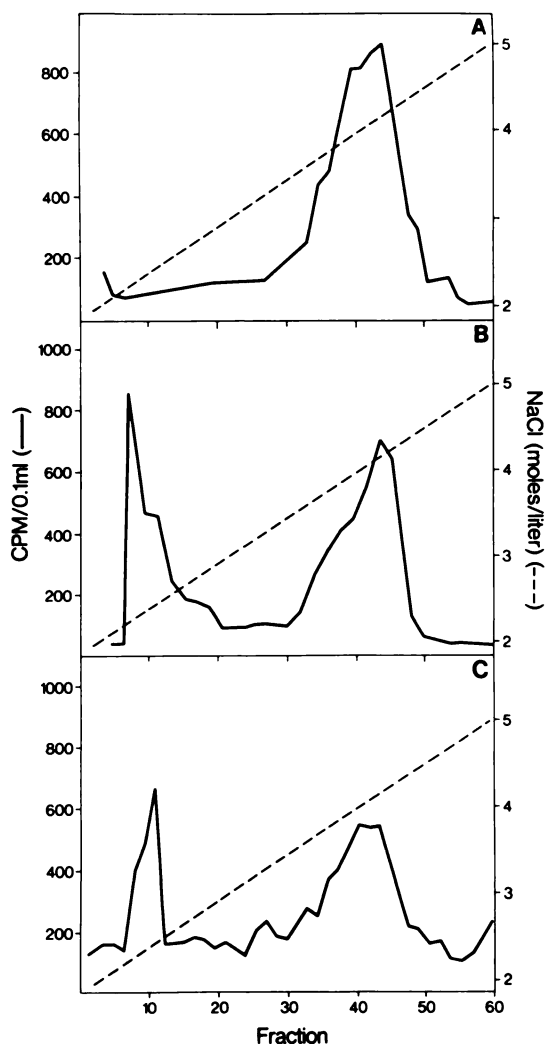


Chart 2. Plasma conjugation of [^{14}C]spermidine 5 (A), 30 (B), and 60 (C) min after injection (see "Materials and Methods"). Radioactivity centered at Fraction 40 corresponds to a [^{14}C]spermidine standard.

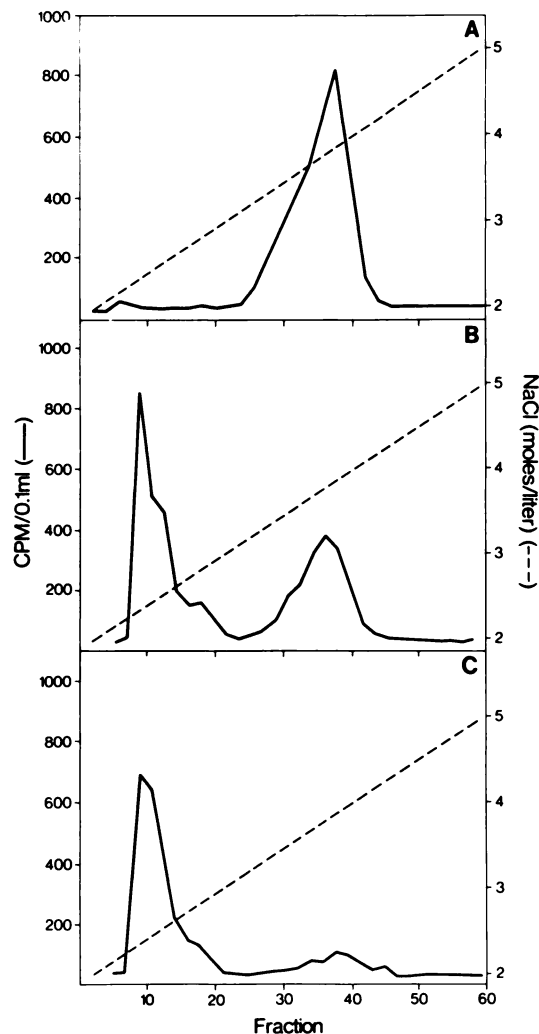


Chart 3. Plasma conjugation of [^{14}C]putrescine at 5 (A), 30 (B), and 60 (C) min after injection (see "Materials and Methods"). Radioactivity centered at Fraction 35 corresponds to a [^{14}C]putrescine standard.

sary for excretion. Clinical evidence indicates that human urinary polyamine levels are best expressed as a function of creatinine (13). The conjugation rates of putrescine and spermidine are compared in Table 1. The higher levels of conjugated putrescine than of conjugated spermidine at 60 min suggest that the lower distribution of putrescine to extravascular sites may make it more available for conjugation.

Effect of Nephrectomy and Hepatectomy of Conjugation of [^{14}C]Spermidine. Table 1 illustrates the conjugation patterns of [^{14}C]spermidine in nephrectomized and hepatectomized animals. After unilateral nephrectomy, the level of spermidine conjugate in the serum was higher than control at 60 min after injection (75% in the conjugate form compared to 50% in control). This suggests that renal excretion is a determinant of either free spermidine levels or, more likely, of conjugated spermidine levels. Hepatectomy totally abolished spermidine conjugation (Table 1). This suggests that conjugation may be a liver-dependent process.

Recovery of [^{14}C]Spermidine and Cold Spermidine after Conjugate Hydrolysis. After partial purification of the [^{14}C]spermidine conjugate from rat urine (see "Materials

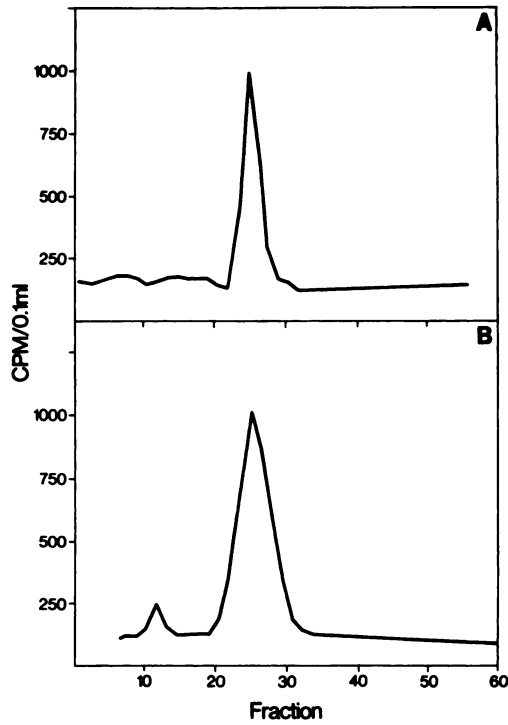


Chart 4. Bio-Rex-70 chromatogram of plasma [¹⁴C]spermine 5 (A) and 60 (B) min after injection (see "Materials and Methods"). Radioactivity centered at Fraction 25 corresponds to a [¹⁴C]spermine standard.

Table 1

Appearance of [¹⁴C]polyamine conjugates in the plasma of rats

Free ¹⁴C and ¹⁴C-labeled conjugated polyamines were determined by Dowex 50 cation-exchange chromatography (see "Materials and Methods") after a 2.5- μ Ci i.v. injection of labeled polyamines. Values are for anesthetized male rats (300 to 400 g) and reflect the mean of duplicate determinations of 4 separate animals.

Treatment	Time (min)	% conjugated
[¹⁴ C]Putrescine	5	10
	15	22
	30	60
	60	90
[¹⁴ C]Spermidine	5	1
	15	10
	30	50
	60	50
[¹⁴ C]Spermine	5	<0.1
	60	<0.1
[¹⁴ C]Spermidine and unilateral nephrectomy	5	2
	15	10
	30	52
	60	75
[¹⁴ C]Spermidine and hepatectomy	5	<0.1
	15	<0.1
	30	<0.1
	60	<0.1

and Methods"), analysis by cation-exchange chromatography (Chart 5) indicated that the spermidine conjugate (Chart 5A) released unmetabolized [¹⁴C]spermidine label after acid hydrolysis. Further analysis of the purified spermidine conjugate on a Durrum D-500 amino acid analyzer confirmed

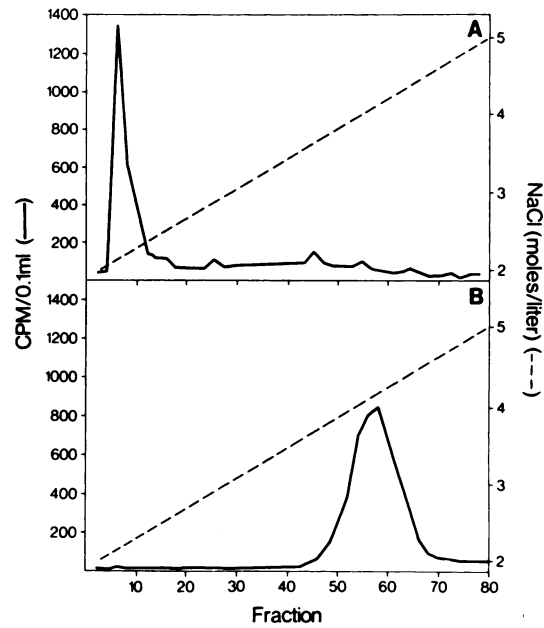


Chart 5. Dowex 50 chromatograms of [¹⁴C]spermidine urine conjugate prior to (A) and after (B) acid hydrolysis. The chromatographic profile in B corresponds to a [¹⁴C]spermidine standard.

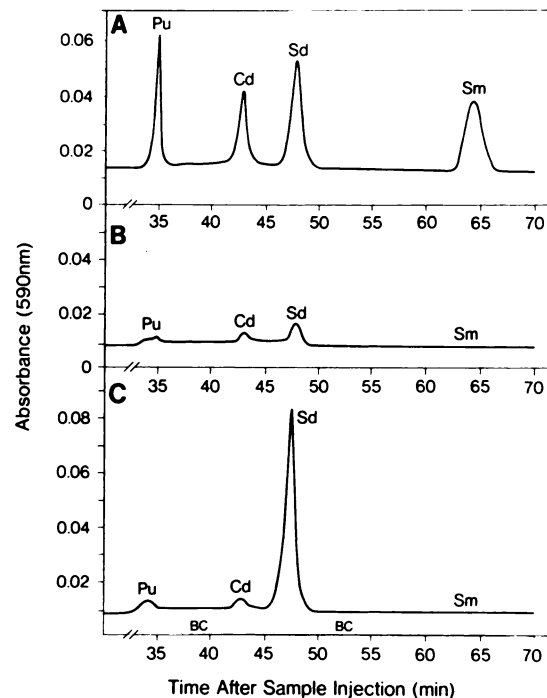


Chart 6. Durrum D-500 amino acid analysis of purified urinary spermidine conjugate (see "Materials and Methods"). A, standard tracings at 590 nm of an aqueous solution of pure putrescine (Pu), cadaverine (Cd), spermidine (Sd), and spermine (Sm). Spermidine conjugate isolated from urine analyzed for free polyamines prior to (B) and after (C) hydrolysis.

that prior to hydrolysis (Chart 6B) there was little free spermidine. An acid hydrolysate, however, contained a 10-fold greater concentration of free spermidine (Chart 6C).

Although polyamine biogenesis has been extensively studied in normal and neoplastic growth, along with the enzymes responsible for polyamine formation, little is known about conjugates of polyamines formed in plasma

and urine. Preliminary evidence from our laboratory indicates that these conjugates are not simply acetyl derivatives which have been reported in bacteria (15) and in the urine of certain cancer patients (2). Gas chromatography-mass spectrometry analysis before hydrolysis shows no acetyl moiety (M. G. Rosenblum, S. Chong, and D. H. Russell, unpublished results). Further, our preliminary amino acid analyses of hydrolysates of the purified spermidine conjugate indicate that our isolated polyamine conjugate is not glutathionyl spermidine which has been isolated from *E. coli* (17), although the conjugate appears to contain several amino acids.

REFERENCES

1. Bachrach, U., and Ben-Joseph, M. Tumor Cells, Polyamines, and Polyamine Derivatives. *In*: D. H. Russell (ed.), *Polyamines in Normal and Neoplastic Growth*, pp. 15-26. New York: Raven Press, 1973.
2. Denton, M. D., Glazer, H. S., Walle, T., Zellner, D. C., and Smith, F. G. Clinical Application of New Methods of Polyamine Analysis. *In*: D. H. Russell (ed.), *Polyamines in Normal and Neoplastic Growth*, pp. 373-380. New York: Raven Press, 1973.
3. Dreyfuss, F., Chayen, R., Dreyfuss, G., Dvir, R., and Ratan, J. Polyamine Excretion in the Urine of Cancer Patients. *Israel J. Med. Sci.*, *11*: 785-795, 1975.
4. Marton, L. J., Russell, D. H., and Levy, C. C. Measurement of Putrescine, Spermidine and Spermine in Physiological Fluids by Use of an Amino Acid Analyzer. *Clin. Chem.*, *19*: 923-926, 1973.
5. Marton, L. J., Vaughn, J. G., Hawk, I. A., Levy, C. C., and Russell, D. H. Elevated Polyamine Levels in Serum and Urine of Cancer Patients: Detection by a Rapid Automated Technique Utilizing an Amino Acid Analyzer. *In*: D. H. Russell (ed.), *Polyamines in Normal and Neoplastic Growth*, pp. 367-372. New York: Raven Press, 1973.
6. Russell, D. H. Increased Polyamine Concentrations in the Urine of Human Cancer Patients. *Nature*, *233*: 144-145, 1971.
7. Russell, D. H. Polyamines in Growth—Normal and Neoplastic. *In*: D. H. Russell (ed.), *Polyamines in Normal and Neoplastic Growth*, pp. 1-14. New York: Raven Press, 1973.
8. Russell, D. H., Durie, B. G. M., and Salmon, S. E. Polyamines as Predictors of Success and Failure in Cancer Chemotherapy. *Lancet*, *2*: 797-804, 1975.
9. Russell, D. H., Gullino, P. M., Marton, L. J., and LeGendre, S. M. Polyamine Depletion of the MTW9 Mammary Tumor and Subsequent Elevation of Spermidine in the Sera of Tumor-bearing Rats as a Biochemical Marker of Tumor Regression. *Cancer Res.*, *34*: 2378-2381, 1974.
10. Russell, D. H., Levy, C. C., Schimpff, S. C., and Hawk, I. A. Urinary Polyamines in Cancer Patients. *Cancer Res.*, *31*: 1555-1558, 1971.
11. Russell, D. H., Looney, W. B., Kovacs, C. J., Hopkins, H. A., Dattilo, J. W., and Morris, H. P. Changes in Serum Putrescine and Spermidine Levels following Local Radiation to Hepatoma 3924A of the Rat. *Cancer Res.*, *36*: 420-423, 1976.
12. Russell, D. H., Looney, W. B., Kovacs, C. J., Hopkins, H. A., Marton, L. J., LeGendre, S. M., and Morris, H. P. Polyamine Depletion of Tumor Tissue and Subsequent Elevation of Spermidine in the Sera of Rats with 3924A Hepatomas after 5-Fluorouracil Administration. *Cancer Res.*, *34*: 2382-2385, 1974.
13. Russell, D. H., and Russell, S. D. Comparison of the Relative Usefulness of Serum, Plasma and Urine Levels of Polyamines as Biochemical Markers of Cancer. *Clin. Chem.*, *21*: 860-863, 1975.
14. Schimpff, S. C., Levy, C. C., Hawk, I. A., and Russell, D. H. Polyamines—Potential Roles in the Diagnosis, Prognosis and Therapy of Patients with Cancer. *In*: D. H. Russell (ed.), *Polyamines in Normal and Neoplastic Growth*, pp. 395-404. New York: Raven Press, 1973.
15. Tabor, C. W. The Effect of Temperature on the Acetylation of Spermidine. *Biochem. Biophys. Res. Commun.*, *30*: 339-342, 1968.
16. Tabor, H., and Tabor, C. W. Spermidine, Spermine and Related Amines. *Pharmacol. Rev.*, *16*: 245-300, 1964.
17. Tabor, H., and Tabor, C. W. Glutathionyl Spermidine in *Escherichia coli*. *Ital. J. Biochem.*, *25*: 70-76, 1976.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Conjugation of Radiolabeled Polyamines in the Rat

Michael G. Rosenblum and Diane H. Russell

Cancer Res 1977;37:47-51.

Updated version Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/37/1/47>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link <http://cancerres.aacrjournals.org/content/37/1/47>. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.