

# Cardiac Stimulant Action of Constituents of *Aloe Saponaria*

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**Abstract** □ A highly potent cardiotoxic substance, calcium isocitrate, was isolated from *Aloe saponaria*, using solvent partition, nonionic porous resin, and gel permeation chromatographies. Cardiac stimulant activity of synthesized stereoisomers of calcium isocitrate was demonstrated in isolated guinea pig atria.

**Keyphrases** □ *Aloe Saponaria*—cardiac stimulation activity of various constituents, calcium isocitrate □ Calcium isocitrate—isolated from *Aloe saponaria*, cardiac stimulation of rabbit and rat, and guinea pig atria □ Stereoisomers—synthesis of calcium isocitrate stereoisomers, cardiac stimulation activity, rats, rabbits, guinea pigs

*Aloe* is a group of succulent plants native to dry areas, especially southern Africa. The leaves of *Aloe saponaria* have been used widely as folk medicine for various external and internal diseases such as burns, cuts, ulcers, and stomach disorders, etc. Aloin has been isolated from the plants as the major component (1) and is used as a purgative. While examining the antibacterial and antitumor activities of *Aloe* extracts (2), the cardioexcitatory effect of the extracts on the isolated atria was recently identified. The present experiments were undertaken to isolate the cardiotoxic substances from *A. saponaria* and to evaluate their positive inotropic effects on isolated cardiac muscles.

## EXPERIMENTAL

**Materials**<sup>1</sup>—Nonionic porous resin<sup>2</sup>, anion exchange resin<sup>3</sup>, dextran gel<sup>4</sup>, dextran gel possessing both hydrophilic and lipophilic properties<sup>5</sup>, and thin layer chromatography (TLC) aluminum sheets of silica gel<sup>6</sup> were purchased from suppliers. TLC was performed using butanol-ethanol-chloroform-28% ammonium hydroxide (4.5:2:8) and ethyl acetate-butanol-acetic acid-water (6:8:5:8) as solvent systems. Anisaldehyde-sulfuric acid and iodine were used as spraying reagents.

**Chromatography**—The flow rate for a column of resin<sup>2</sup> (4 × 6 cm) was ~20 ml/hr and for resin<sup>3</sup> (1.5 × 15 cm) was ~20 ml/hr. The gel filtration through dextran gel<sup>4</sup> (2.5 × 36 cm) and gel<sup>5</sup> (1.5 × 40 cm) was performed at room temperature at a flow rate of 21 ml/hr. The chromatography with silica gel<sup>6</sup> was performed through a column (2 × 22 cm).

**Bioassay**—Bioassay of compounds was performed on isolated atria of rat, rabbit, and guinea pig hearts. The atrium was separated from the rest of the heart and suspended in a 50-ml tissue bath containing Krebs-Ringer bicarbonate solution of the following composition (mM): sodium chloride, 120.3; potassium chloride, 4.8; calcium chloride, 1.2; magnesium sulfate, 1.3; potassium biphosphate, 1.2; sodium bicarbonate, 25.2; and glucose 5.5. The solution was continuously bubbled with a gas mixture of 95% oxygen and 5% carbon dioxide and maintained at 30°, pH 7.4. Tension was recorded isometrically through a force displacement

transducer<sup>7</sup> and displayed on a polygraph<sup>8</sup>. In some experiments, spontaneously beating right atria were used; in other cases, the left atria were driven by an electrical stimulator<sup>9</sup> at a frequency of 1.0 Hz with square wave pulses of 5 msec at 100% above threshold voltage. The atria were allowed to equilibrate under 0.5-g tension for 60 min prior to beginning the experiment. The changes in contractile force and rate produced by the test agents are expressed as a percentage variation.

**Extraction**—The fresh leaves of *Aloe saponaria*<sup>10</sup> were harvested from the greenhouse of the herbal garden of Kyushu University. Fresh leaves (20 kg) were cut in half and colorless gelatinous pulp was separated carefully by scraping the green cortical layer which contained yellow phenolics. The homogenized pulp (6 kg) was extracted with distilled water, filtered, and the filtrate centrifuged at 10,000 rpm for 30 min. The supernate was dialyzed by dialysis membrane<sup>11</sup> against distilled water for 48 hr. The dialysate was flash evaporated at ≤50° to dryness (50 g).

**Chromatography on Nonionic Porous Resin**—The dialysate (4.4 g) suspended in methanol was chromatographed on a column of resin<sup>2</sup> (150 ml). After the methanolic eluate containing sugar moiety was excluded, the cardiac stimulant fraction was eluted with methanol-water (1:5) and water and evaporated to dryness.

**Gel Permeation Chromatography**—The crude extract dissolved in a small amount of water was reprecipitated by adding methanol to yield a colorless powder A. The colorless powder A (1 g) was chromatographed over dextran gel<sup>4</sup> (400 ml), using 3 ml of water as a solvent, and fractionated to fraction A (tube numbers 1-4) and Fraction B (tube numbers 5-10). Each fraction was flash evaporated to dryness and monitored by TLC. Fraction B (0.8 g) was further chromatographed over dextran gel<sup>4</sup> (400 ml), using 3 ml of water as a solvent, and fractionated to Fraction C (tube numbers 5-9, 0.3 g), and Fraction D (tube numbers 10-15, 0.2 g). Each fraction was flash evaporated to dryness and monitored by TLC.

**Characterization of Compound I**—Fraction C was reprecipitated from methanol to give a colorless powder, compound I, mp >290°, which had a strong absorption band due to carboxylate ion on IR spectrum, and presented a positive reaction to calcium ion (calcium oxalate). Compound I (1 g), dissolved in water (10 ml), was put on an anion exchange resin<sup>3</sup> column (20 ml) and eluted with water. The elution was continued until no residue was obtained in the flash evaporation. Then, 10% formic acid was passed through the column and the effluent was evaporated to dryness *in vacuo*. The residue (0.6 g) was chromatographed over dextran gel<sup>5</sup> using methanol as a solvent to give a colorless powder, compound I (free form). PMR (deuterium oxide) δ: 2.72(2H; d, d;  $J_{gem} = 16$ ;  $J = 6, 10$  Hz;  $-CH_2-CH_2-$ ), 3.36(1H, m,  $-CH_2-CH-$ ), 4.40(1H, d,  $J = 4$  Hz,  $-CHOH-CH-$ ). CMR (deuterium oxide) δ: 34.8(t,  $-CH_2-$ ), 47.5(d,  $-CH-$ ), 72.8(d,  $-CHOH-$ ), 176.0, 179.0(s, COOH × 3). Compound I (free form, 40 mg) dissolved in methanol (10 ml) was methylated with diazomethane under ice cooling for 1 hr, and the reaction mixture was evaporated to dryness to yield a yellow oil (47 mg). The product was chromatographed over silica gel<sup>7</sup> using the solvent, ethyl acetate-chloroform (1:99), to give a colorless oil of the methyl ester (40 mg).  $[\alpha]_D^{25} + 4.8^\circ$  (c, 2.0 in chloroform). PMR (deuteriochloroform) δ: 2.62(1H, d, d,  $J_{gem} = 18$ ,  $J = 8$  Hz,  $-CH_2-CH-$ ), 2.90(1H, d, d,  $J_{gem} = 18$ ,  $J = 8$  Hz,  $-CH_2-CH-$ ), 3.24(1H, d,  $J = 6$  Hz,  $-CHOH-$ ), 3.48(1H; sex;  $J = 8, 3$  Hz;  $-CH-CH-CH_2-$ ), 3.64, 3.66, 3.78(COOCH<sub>3</sub> × 3), 4.32(1H; q;  $J = 6, 3$  Hz;  $-CHOH-CH-$ ). CMR (deuterium oxide) δ: 32.11(t,  $-CH_2-$ ), 44.88(d,  $-CH-$ ), 51.85, 52.15, 52.73(q, COOCH<sub>3</sub>), 70.47(d,

<sup>7</sup> Grass model FT-03.

<sup>8</sup> Grass polygraph.

<sup>9</sup> Grass SD-5.

<sup>10</sup> A voucher specimen is available for inspection at Higashiyama Botanical Garden, Nagoya, Japan.

<sup>11</sup> Visking Co.

<sup>12</sup> Dowex 50W<sub>4</sub>, Dow Chemical Co.

<sup>1</sup> IR, PMR (tetramethylsilane as the internal standard), CMR (tetramethylsilane as the internal standard) and mass spectra were recorded using KOKEN DS-301, JEOL PS-100, JEOL FX-100, and JMS D-300, respectively. In the PMR descriptions, s = singlet, d = doublet, d,d = doublet of doublets, t = triplet, q = quartet, sex = sextet, and m = multiplet. Optical rotations were obtained on a JASCO DIP-4 and melting points were determined on a Yanagimoto melting point apparatus and are uncorrected.

<sup>2</sup> Amberlite XAD-2, Rohm and Hass Co. Ltd.

<sup>3</sup> Amberlite IRA-400 (OH<sup>-</sup>), Rohm and Hass, Co. Ltd.

<sup>4</sup> Sephadex G-50, Pharmacia Fine Chemicals.

<sup>5</sup> Sephadex LH-20, Pharmacia Fine Chemicals.

<sup>6</sup> Merck 60.

**Table I—Positive Inotropic Effect of Fractions of Aloe Extracts on Isolated Atria**

Fraction	Increase, % mean, (n = 2-3)	Concentration, mg/ml	Animal
Dialysate	35.8	0.5	rabbit
Nonionic porous resin <sup>2</sup> methanol-water (1:5) water	550 570	1.0 1.0	rabbit rabbit
Powder A	350	1.0	rat <sup>b</sup>
Fraction A <sup>c</sup>	220	1.0	rat
Fraction C <sup>a,d</sup>	243	3.0	rat
Fraction D <sup>a,e</sup>	88	3.0	rat
Isoproterenol	320	5 × 10 <sup>-8</sup> M	rabbit
Isoproterenol	250	5 × 10 <sup>-8</sup> M	rat

<sup>a</sup> Fraction C and D increased the heart rate 23.0–55.3%. <sup>b</sup> Tissues were beating spontaneously. Similar results were also observed on the electrically driven atria (1.0 Hz). <sup>c</sup> Fraction A: Mixture of calcium isocitrate, malate, succinate, etc. <sup>d</sup> Fraction C: Calcium isocitrate. <sup>e</sup> Fraction D: Calcium malate.

**Table II—Positive Inotropic Effect of Various Synthesized Isocitrates on Guinea Pig Atria<sup>a</sup>**

Compound <sup>b</sup>	Increase, % <sup>c</sup>	Concentration, mg/ml
(+)-Calcium isocitrate	30 ± 4	5 × 10 <sup>-2</sup>
	129 ± 16	10 <sup>-1</sup>
(±)-Calcium isocitrate	90 ± 8	5 × 10 <sup>-2</sup>
	171 ± 18	10 <sup>-1</sup>
(±)-Calcium allosictrate	115 ± 15	5 × 10 <sup>-2</sup>
	148 ± 8	10 <sup>-1</sup>
(+)-Calcium isocitrate <sup>d</sup>	132 ± 10	10 <sup>-1</sup>
Isoproterenol	330 ± 35	5 × 10 <sup>-8</sup> M

<sup>a</sup> Four atria from different animals were used for each experiment, mean ± SE. <sup>b</sup> All compounds caused no apparent change in heart rate. <sup>c</sup> Increase of norepinephrine, 10<sup>-6</sup> mg/ml, 92%. <sup>d</sup> Isolated from *Aloe saponaria*.

—CHOH—), 170.9, 171.8, 172.9(s, COOCH<sub>3</sub>). Mass spectrum *m/z*: 235(M<sup>+</sup> + 1), 175(M<sup>+</sup> - 60), 143(M<sup>+</sup> - 59, -32), 115(M<sup>+</sup> - 60, -60). The methyl ester was identified with methyl isocitrate (3, 4) by direct comparison (IR and TLC).

**Characterization of Compound II**—Fraction D (0.4 g) was reprecipitated from methanol to give a colorless powder, compound II, mp >290°, which had a strong absorption band due to a carboxylate ion on IR spectrum and presented a positive reaction to calcium ion (calcium oxalate). Compound II (1 g) dissolved in water (10 ml) was put on an anion exchange resin<sup>3</sup> column (20 ml) and eluted with water. The elution was continued until no residue was obtained in flash evaporation. Then, 10% formic acid was passed through the column and the effluent was evaporated to dryness *in vacuo*. The residue (170 mg) was chromatographed over dextran gel<sup>5</sup> (20 ml) using acetone as a solvent to give a colorless powder, compound II (free form, 50 mg) together with a small amount of Compound III and IV (free form). Compound II (free form) mp 101–102° (ethyl acetate), [α]<sub>D</sub><sup>25</sup> -6.0° (C, 0.4 in acetone). IR (potassium bromide) cm<sup>-1</sup>: 2800–2600 (hydroxyl), 1700 (carboxylic acid). PMR (deuteroacetone) δ: 2.74(2H, d; q, *J*<sub>gem</sub> = 16 Hz; *J* = 8, 4 Hz; —CH<sub>2</sub>—CH—), 4.50(1H, d; d; *J* = 8, 4 Hz; —CH—CH<sub>2</sub>—), 7–8(3H, OH, COOH). Compound II (free form, 10 mg) dissolved in methanol (10 ml) was methylated with diazomethane under ice cooling for 1 hr, and the reaction product (12 mg) was chromatographed over silica gel<sup>6</sup> using the solvent, *n*-hexane-chloroform (1:1) to yield a colorless oil of methyl ester (10 mg). [α]<sub>D</sub><sup>25</sup> -2.5° (C, 0.4 in chloroform). IR (liquid film) cm<sup>-1</sup>: 3350 (hydroxyl), 1730(ester), 1440(methylene), 1100. PMR (deuteriochloroform) δ: 2.66(1H, d, d, *J*<sub>gem</sub> = 16, *J* = 5 Hz, —CH<sub>2</sub>—), 3.25(1H, OH), 3.71, 3.81 (COOCH<sub>3</sub> × 2), 4.50(1H, d; d; *J* = 5, 5 Hz; —CHOH—CH<sub>2</sub>—). CMR (deuteriochloroform) δ: 38.38(—CH<sub>2</sub>—), 51.91, 52.68(COOCH<sub>3</sub> × 2), 67.09(CHOH), 170.56, 173.32(COOH × 2). Mass spectrum *m/z*: 163(M<sup>+</sup> + 1), 103(M<sup>+</sup> - 59). The methyl ester was identified with methyl malate by direct comparison (IR and TLC).

**Characterization of Compound III**—Compound III (free form) was obtained from Fraction D, which showed a positive reaction to calcium ion (calcium oxalate) by the following procedure for the isolation of Compound I (free form): mp 185° (acetone), IR (potassium bromide) cm<sup>-1</sup>: 2800–2600(hydroxyl), 1700(carboxylic acid). PMR (deuterio-methanol) δ: 2.56(2H, s, —CH<sub>2</sub>—). CMR (deuterio-methanol) δ: 29.77(—CH<sub>2</sub>—), 175.78(COOH). Compound III (free form) was identified with succinic acid by direct comparison (IR and mixed melting point).

**Characterization of Compound IV**—Compound IV (free form) was obtained from the Fraction D, which showed a positive reaction to calcium ion (calcium oxalate) by the following procedure for the isolation of compound I (free form): mp 74–75° (ethyl acetate), [α]<sub>D</sub><sup>20</sup> -6.8° (C, 6.6 in acetone), IR (potassium bromide) cm<sup>-1</sup>: 2800–2600(hydroxyl), 1725(ester), 1440(methylene). PMR (deuteriochloroform) δ: 2.86(2H; d; d; *J*<sub>gem</sub> = 16; *J* = 8, 6 Hz; —CH<sub>2</sub>—CH—), 2.80(3H, COOCH<sub>3</sub>), 4.52(1H; q; *J* = 8, 6 Hz; —CH<sub>2</sub>—CH—), 6.34(br, s, OH). CMR (deuterio-methanol) δ: 38.32(—CH<sub>2</sub>—), 52.80(COOCH<sub>3</sub>), 66.97(CHOH), 173.37(COOCH<sub>3</sub>), 174.60(COOH). Mass spectrum *m/z*: 149(M<sup>+</sup> - 17), 103(M<sup>+</sup> - 45), 89(M<sup>+</sup> - 59). Based on the comparative study of CMR spectra the structure of compound IV (free form) was determined to be (–)-2-hydroxybutandiolic acid 4-methyl ester.

**Syntheses of (±), (+) or (–)-Calcium Isocitrate and of (±)-Calcium Allosictrate**—The following compounds were synthesized according to the literature (3):

(±)-Isocitric Acid Lactone—mp 156–158° (ethyl acetate), PMR (deuterodimethyl sulfoxide) δ: 2.76(2H, d, *J* = 8 Hz), 3.70(1H, sex; *J* = 7.5, 8 Hz), 5.11(1H, d, *J* = 7.5 Hz), 10.5(2H, br).

(+)-Isocitric Acid Lactone—mp 152–153° (ethyl acetate), [α]<sub>D</sub><sup>25</sup> +59.4° (C, 1.3 in water).

(–)-Isocitric Acid Lactone—mp 151–153° (ethyl acetate), [α]<sub>D</sub><sup>25</sup> -53.0° (c, 1.1 in water).

(±)-Alloisocitric Acid Lactone—mp 155–157° (ethyl acetate), PMR (deuterodimethyl sulfoxide) δ: 2.75(2H, d, *J* = 7.5 Hz), 3.45(1H, m), 5.05(1H, d, *J* = 4.0 Hz), 9.70(2H, br).

(±)-Isocitric acid lactone (2.0 g) dissolved in 2 N NaOH (20 ml) was hydrolyzed at 90° for 3 hr and the reaction mixture was passed through cation exchange resin<sup>12</sup> (70 ml). Saturated calcium hydroxide solution was added to acidic eluate and the pH of the solution was made up to 9.0. After the filtration, the filtrate (200 ml) was concentrated to a 20 ml volume, and from the precipitate (±)-calcium isocitrate (2.78 g) was obtained. IR (potassium bromide) cm<sup>-1</sup>: 3400, 1635, 1600, 1570, 1400, 1310, 1110, 1070. PMR (deuterium oxide-deuterium chloric acid) δ: 2.79(2H, d, *J* = 6.5 Hz), 3.46(1H, m), 4.50(1H, d, *J* = 3.9 Hz). Quantitative analysis of calcium ion for (C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>)<sub>2</sub>Ca<sub>3</sub>·5H<sub>2</sub>O: Calc. 20.43%. Found: 20.54%. In the same way as the formation of (±)-calcium isocitrate, (+), (–)-calcium isocitrate and (±)-calcium allosictrate were synthesized.

## RESULTS AND DISCUSSION

Table I summarizes the cardiac stimulatory action (inotropic) of each fraction of *Aloe saponaria* extract after dialysis and chromatography using resin<sup>2</sup> and dextran gel<sup>4</sup>. The dialysate caused a positive inotropic action on isolated rabbit atria without any change in the diastolic tension. Pretreatment of atria with propranolol (10<sup>-6</sup> M, a β-adrenoreceptor blocking agent) or phenolamine (10<sup>-6</sup> M, an α-adrenoreceptor blocking agent) had no effect on the inotropic response to the dialysate. Thus, it is apparent that the cardiac stimulatory action of the dialysate and other extracts are not mediated through the stimulation of adrenergic receptors.

In the preliminary examinations on cellulose and silica gel chromatographs, using butanol-acetic acid-water (4:1:5) and butanol-ethanol-chloroform-28% ammonium hydroxide (4:5:2:8) as solvents, the inotropic substance in the dialysate was demonstrated to be a hydrophilic substance which is considerably stable for acid, alkaline, and heat treatments. Since the methanol eluate on resin<sup>2</sup> column chromatography failed to cause any inotropic effect, the fraction was completely excluded from active substances. Active components were obtained from the effluent of methanol-water (1:5) and water. Then, the gel filtration using dextran gel<sup>4</sup> was performed to give fractions of A, C, and D. From these fractions compounds I–IV were obtained. Each compound showed a single spot on TLC-2 and was determined to be a calcium salt of organic acid by the positive reaction to calcium ion. On the treatment with anion exchange resin<sup>3</sup>, compound I or II provided an organic acid in the free form which did not cause any cardiac stimulation on the isolated rat atria. Thus, it is suggested that compounds I and II have cardiac stimulation action only in the form of calcium salts. The chemical and spectral examinations led to the conclusion that compounds I–IV are calcium salts of (+)-isocitric (3, 4), L-(–)-malic, succinic, and of (–)-2-hydroxybutandiolic acid 4-methyl ester, respectively. Calcium salts of the stereoisomers of isocitric acid were synthesized according to the literature (3) and the cardiotoxic effect of these synthetic isomers and compound I on isolated atria were

carried out. As shown in Table II, calcium salts of (+), (±)-isocitric acid and (±)-alloisocitric acid caused a positive inotropic effect on isolated guinea pig atria to the same degree as compound I at a concentration of  $10^{-4}$  g/ml, while no toxic effect such as arrhythmia was observed.

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# Antitumor Agents LIII: The Effects of Daphnoretin on Nucleic Acid and Protein Synthesis of Ehrlich Ascites Tumor Cells

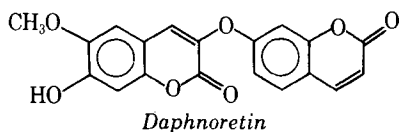
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**Abstract** □ Daphnoretin, a dicoumaryl ether, has been shown to inhibit growth of Ehrlich ascites carcinoma cells. Dosing for three days at 6 mg/kg/day reduced the cell number per milliliter by 60%. *In vitro* DNA and protein synthesis studies demonstrated an  $ID_{50} \approx 0.194$  mM and  $ID_{50} \approx 0.340$  mM, respectively, for daphnoretin. Subsequently it has been shown that *in vivo* nucleic acid and protein synthesis were inhibited. Major sites in the DNA synthetic pathway inhibited significantly by daphnoretin were dihydrofolate reductase, orotidine monophosphate decarboxylase, thymidylate monophosphate kinase, and ribonucleotide reductase. Reduction of *in vitro* oxidative phosphorylation processes and acid hydrolytic enzymes were also inhibited in the presence of daphnoretin.

**Keyphrases** □ Daphnoretin—effects on nucleic acid and protein synthesis of Ehrlich ascites tumor cells, antitumor agent □ DNA—effects of daphnoretin, protein synthesis, Ehrlich ascites tumor cells □ Protein synthesis—effects of daphnoretin, DNA, Ehrlich ascites tumor cells □ Antitumor agents—the effects of daphnoretin on nucleic acid and protein synthesis of Ehrlich ascites tumor cells

Daphnoretin has been isolated from the whole plant of *Wikstroemia indica* C. A. Mey (Thymelaeaceae) (1), which is known as “Nan-Ling-Jao-Hua” or “Po-Lun” in Chinese folklore as a herbal remedy for the treatment of human syphilis, arthritis, whooping cough (2), and cancer (3). Daphnoretin was shown to have significant inhibitory activity *in vivo* against the Ehrlich ascites carcinoma growth in mice but did not demonstrate any activity against P-388 lymphocytic leukemia growth (4). A detailed examination of the effects of daphnoretin on nucleic acid and protein synthesis of Ehrlich ascites carcinoma cells is presented.



## EXPERIMENTAL

CF<sub>1</sub> male mice (~30 g) were implanted with  $2 \times 10^6$  Ehrlich ascites tumor cells intraperitoneally on day 0. Daphnoretin was suspended by

homogenization in 0.05% polysorbate 80–water and 3–12 mg/kg ip was administered for 9 days to determine the inhibition of tumor growth. Mice were sacrificed on day 10, and the ascites fluid was collected from the peritoneal cavity. The volume and ascites were determined for each animal and the inhibition of tumor growth was calculated (5). For the metabolic studies, mice were treated on days 7–9 with 6 mg/kg ip of daphnoretin. The animal was sacrificed on day 10 and the ascites fluid was harvested. The *in vitro* metabolic studies were performed at 0.340 mM.

*In vitro* incorporation of [<sup>3</sup>H]thymidine, [<sup>3</sup>H]uridine, or [<sup>3</sup>H]leucine was determined using  $10^6$  Ehrlich ascites cells, 1  $\mu$ Ci labeled precursor, minimum essential medium, and varying final concentrations of drug from 0.035 to 0.35 mM. The tubes were incubated at 37° for 60 min and inactivated by trichloroacetic acid. The acid insoluble-labeled DNA was collected on glass filter discs<sup>1</sup>, and RNA and protein were precipitated on nitrocellulose filters by vacuum suction (6). Results are expressed as disintegrations per minute of incorporated precursor per hour per  $10^6$  ascites cells.

For *in vivo* studies, incorporation of thymidine into DNA was determined by the method of Chae *et al.* (7). One hour prior to the animal sacrifice on day 10, 10  $\mu$ Ci of [6-<sup>3</sup>H]thymidine (21.5 Ci/mole, ip) were injected. The DNA was isolated and the tritium content was determined in a toluene based scintillation fluid<sup>2</sup>. The DNA concentration was determined by the diphenylamine reaction using calf thymus DNA as a standard. Uridine incorporation into RNA was determined using 10  $\mu$ Ci of [5,6-<sup>3</sup>H]uridine (22.4 Ci/mole). RNA was extracted by the method of Wilson *et al.* (8). Using yeast RNA as a standard, the RNA content was assayed by the orcinol reaction. Leucine incorporation into protein was determined by the method of Sartorelli (9) using 10  $\mu$ Ci of [4,5-<sup>3</sup>H]leucine (52.2 Ci/mole). Protein content was determined by the Lowry procedure using bovine serum albumin as a standard.

*In vitro* and *in vivo* nuclear DNA polymerase activity was determined on isolated Ehrlich ascites cell nuclei (10). The incubation was that described previously (11), except that [methyl-<sup>3</sup>H]deoxythymidine triphosphate (82.4 Ci/mole) was used. The acid insoluble nucleic acid was collected on filters and counted. Nuclear RNA polymerase activities were determined on enzymes isolated from nuclei. Messenger, ribosomal, and transfer RNA polymerase enzymes were isolated using 0.3, 0.04, and 0.0 M concentrations, respectively, of ammonium sulfate in magnesium chloride. The incubation medium was [<sup>3</sup>H]uridine triphosphate (23.2 Ci/mole) (12). The acid insoluble RNA was collected on nitrocellulose filters and counted.

Deoxythymidine, deoxythymidylate monophosphate, and diphosphate kinase activities were measured spectrophotometrically at 340 nm at 20

<sup>1</sup> GF/F glass filter discs.

<sup>2</sup> Fisher Scintiverse.