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Distribution and Valence State of Radiochromium in Intracellularly Labeled Ehrlich Mouse Ascites Carcinoma Cells.* (24298)

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Gray and Sterling(1) reported a method for intracellular labeling of human erythrocytes with radioactive sodium chromate ($Na_2Cr^{51}O_4$). This method has been further studied and applied by several workers(2,3), resulting in adoption of the modified procedure as a standard method for study of erythrocyte life span and related investigations(4). Any procedure resulting in an intracellular label, stable for the life of the cell, has potentialities for the study of cellular biology. Thus, in addition to those used for erythrocytes, similar methods have been applied successfully to leucocytes(5) and platelets(6). Recently, the use of radiochromate for labeling cells of Ehrlich mouse ascites carcinoma has been reported(7). It was shown that the label was retained intracellularly until irreversible rupture of the cell occurred, and that incorporation of chromate ions in the cells did not impair their ability to produce subcutaneous tumors in mice, provided a certain maximum concentration of chromate ion was not exceeded. In their original paper, Gray and Sterling(1) studied the nature of intracellular label in erythrocytes, and demonstrated that the erythrocyte membrane allowed passage of the CrO_4'' ion, but was selectively impermeable to trivalent chromic (Cr^{+++}) ion. Radioactivity within the red cell was shown to be associated with the globin moiety of the hemoglobin molecule. It was also suggested, on the basis of indirect evidence, that the CrO_4'' ion was intracellularly reduced to the trivalent Cr^{+++} ion, and that the stability of the label was dependent on the firm binding of the Cr^{+++} ion to intracellular protein. Tumor cells are considerably more complex entities than the highly specialized erythrocyte. A study of the valence state and intracellular distribution of radiochromium in these cells was considered of interest, and is the subject of this report.

Methods. Propagation, harvesting and standardization of tumor cells. Ehrlich ascites tumor cells were propagated, harvested, freed of erythrocytes and standard suspensions prepared, by methods described elsewhere(8). *Labeling of tumor cells with radiochromate.* A suspension of 5 x 10^8 tumor cells in 10 ml 0.85% NaCl was labeled as previously described(7), using 0.9 μc of $Na_2Cr^{51}O_4$ (specific activity 39.38 mc/mg, Na_2CrO_4 concentration 0.02 mg/ml; obtained from Abbott Laboratories under the trade name Rachromate). Unincorporated radiochromate was removed by washing thrice with 0.85% NaCl (35 G, 0°C, 5 min). *Fractionation of tumor cells.* The labeled tumor cell suspension was fractionated by a method based on that of Hogeboom and Schneider(9). Cells were suspended in 0.25 M sucrose solution and disrupted by grinding in a Potter-Elvehjem homogenizer (60 min, 0°C). An aliquot of the total homogenate was removed for radioassay. The remainder was layered on 0.34 M sucrose solution and centrifuged (450 G, 0°C, 15 min). The supernate (SI) was reserved, the sediment suspended in 0.25 M sucrose solution, layered on 0.34 M sucrose solution and centrifuged (800 G, 0°C, 15 min). The sediment, consisting largely of nuclei, was dispersed in sodium desoxycholate solution, and aliquots prepared for radiassay; the supernate (SII) was combined with the original supernate (SI). The combined supernates,

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representing the cytoplasmic fraction, were centrifuged (13,000 G 0°C, 30 min). The sediment, consisting of mitochondria and microsomes, was dispersed as above, and aliquots prepared for radioassay. The clear supernate (SIII), containing soluble protein and other metabolites, was divided into 4 portions. The first portion was treated with trichloroacetic acid (final concentration 5%), and the second with absolute ethanol (final concentration 50%) for 1 hour at 37°C. The precipitated protein in each case was separated by centrifugation (900 G, 0°C, 15 min) and dissolved in 10 M urea solution; aliquots of these solutions, and of the respective supernates, were prepared for radioassay. The third portion of the supernate (SIII) was deproteinized with ethanol as described above and the protein-free supernate dialyzed exhaustively against distilled water (30 bag revolutions/minute, 24 hours). Aliquots of the dialyzed material were used for radioassay. *Determination of valence state of intracellular chromium.* The final portion of the supernate (SIII) was used for this purpose. The method was based on that used by Cunningham, McGuir, and Clement(10). Following ethanol precipitation, one ml of a 5% solution of carrier (nonradioactive) chromic chloride ($CrCl_3$) was added and, after equilibration of radioactive and carrier Cr^{+++} ions, both were precipitated with ammonium hydroxide. The precipitate was washed with distilled water (100 G, 0°C, 5 min) and redissolved in 1N HCl. Aliquots of the solution of the precipitate, and also of the combined supernate and washings, were used for radioassay. *Method of radioassay.* The number of counts/minute for all samples were determined in a well-type scintillation counter (Nuclear Model DS-3).

Results. The following results are based on average figures from 3 experiments. The total homogenate obtained from 5 x 10^8 tumor cells labeled with 0.9 μc of $Na_2Cr^{51}O_4$ (approximately 88,000 counts/minute) assayed 18477 counts/minute. This corresponds to approximately 21% uptake, a value which is in accord with previously published results(7). Fractionation of the homogenate into nuclear and mitochondrial-microsomal fractions showed that 17 and 12% respectively of the radioactivity of total homogenate were associated with these fractions. Thus, 71% of the total intracellular activity was present in the soluble cytoplasmic fraction.

TABLE I. Intracellular Distribution of Radiochromium in Ehrlich Mouse Ascites Tumor Cells.

Fraction	Radiochromium Counts/min.*	% uptake*	Avg % uptake
Total homogenate	20,517	100	100
	20,344	100	
	18,402	100	
Nuclear fraction	3,936	19	17
	3,560	17	
	2,984	16	
Mitochondrial-microsomal fraction	2,673	13	12
	2,530	12	
	2,205	12	
Soluble protein†	7,775	38	36
	7,055	35	
	6,582	36	
Protein-free supernate†	5,961	29	29
	6,251	31	
	6,405	28	

* Results of individual experiments.
† Ethanol precipitation.

Trichloroacetic acid precipitation of supernate SIII indicated that 2450 counts/minute (13%) were present in the protein fraction, and 8822 counts/minute (45%) in the protein-free supernate. When ethanol was added as the precipitating agent, 36% of radioactivity was associated with the protein fraction, and 29% with the protein-free supernate. Percentage figures so far presented refer to per cent radioactivity of total homogenate. Expressed in terms of total radioactivity of supernate SIII, ethanol precipitated 52% with protein, while 46% remained in the supernate. Results of individual experiments are summarized in Table I.

Dialysis of the protein-free supernate (ethanol precipitation) showed that, of 6205 counts/minute before dialysis, only 70 counts/minute remained after dialysis (1% of radioactivity of undialyzed protein-free supernate).

Results of the determination of the amount of intracellular chromium present as Cr^{+++} ion are presented in Table II. Practically all the radioactivity of the protein-free supernate was precipitable as Cr^{+++} ion.

Discussion. An examination of the results

TABLE II. Proportion of Radioactivity of Protein-Free Supernate Due to Cr^{51+++} Ion.

Fraction	Counts/min.*	% of total*	Avg % of total
Protein-free supernate	7,426	100	100
	8,048	100	
	6,488	100	
Chromic hydroxide precipitate	6,929	93	94
	7,702	95	
	6,188	95	
Supernate and washings	434	6	5
	292	4	
	257	4	

* Results of individual experiments.

indicated that, in accordance with results previously obtained, tumor cells allow passage of CrO_4'' ions into the cell. Once within the cell, however, hexavalent chromium (CrO_4'') was apparently reduced to the trivalent chromic form (Cr^{+++}). Since the procedure used for precipitation of Cr^{+++} ions from the protein-free supernate involved the possibility of solution of a small portion of the precipitated chromic hydroxide in the first excess of ammonium hydroxide, it is probable that the small amount of total radioactivity not precipitable as Cr^{+++} may be accounted for in this manner. Since these results are in accord with observations on the nature of intracellular label in erythrocytes, and since the tumor cell is permeable to CrO_4'' ions, it appears unlikely that both valence states of chromium coexist in the cell.

The discrepancy between results of trichloroacetic acid and ethanol precipitation of protein was explainable on the basis of the low pH associated with trichloroacetic acid precipitation, since this may bring about release of portein-bound Cr^{+++} ions into the supernate. Ethanol was therefore considered to be the precipitant of choice, and results obtained by alcohol precipitation considered the more reliable.

Part of the radioactivity of nuclear and mitochondrial-microsomal fractions was undoubtedly due to entrained or adsorbed radioactive cytoplasmic protein, and part to nonspecific adsorption of free Cr^{+++} ions to the protein surface components of the particulates in these fractions. In the case of the nuclear fraction, a portion of the radioactivity was probably concentrated in the small number of unbroken cells which are invariably carried down in this fraction. No attempts were made to investigate the possibility of a specific concentration of radiochromium within nuclei, mitochondria, or microsomes.

A point not previously examined, to our knowledge, is that although a large percentage of intracellular Cr^{+++} ion is associated with soluble cytoplasmic protein, a significant amount may be present as unbound Cr^{+++} ion, at least in Ehrlich tumor cells, thus suggesting that the cell membrane may be impermeable to intracellular chromic ions. This suggests that the stability of the intracellular label may not be entirely dependent on protein binding, but is also influenced by membrane permeability. This conclusion is strengthened by direct evidence showing that Cr^{+++} ions do not penetrate the erythrocyte membrane(1), and that radioactivity is almost entirely retained within Ehrlich tumor cells until they are lysed by sonic or other disruption, when almost all of it is released(7).

Summary. 1. Ehrlich mouse ascites carcinoma cells were labeled with radiochromate ($Na_2Cr^{51}O_4$), and the intracellular distribution and valence state of radiochromium studied. 2. Of the total intracellular radioactivity, 71% was associated with the soluble cytoplasmic fraction; of radioactivity of this fraction, ethanol precipitated 52% with the proteins, and 46% remained in the supernate from ethanol precipitation in dialyzable form. 3. Hexavalent chromium ($Cr^{51}O_4''$) appeared to be intracellularly reduced to the trivalent state (Cr^{51+++}). 4. The results suggest that the cell membrane of the Ehrlich ascites tumor cell is impermeable to intracellular Cr^{+++} ions, and that stability of the intracellular label is not dependent on protein binding alone.

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Impairment of Innate Resistance by Triiodothyronine.* (24299)

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Lurie and Ninos(1) observed that rabbits treated with triiodothyronine displayed an enhanced resistance to chronic infection by tubercle bacilli. This paper reports the effect of triiodothyronine on experimental infection of mice by a strain of group A streptococci, by *Candida albicans*, and on transplantable mouse leukemia.

Materials and methods. L-3,3',5 triiodothyronine (Smith, Kline & French Co.) was prepared for inoculation of mice by dissolving 4 mg in 1.3 ml 0.01 N NaOH, 90 mg of NaCl added, and total volume of solution brought to 10 ml with distilled water free of carbonate. Triiodothyronine solution (T3) after sterilization by autoclaving for 30 minutes at 110°C., was stable for 6 days at 4°C. *Streptococcus pyogenes*, type 18, and *Candida albicans*, strain 780, were grown and prepared for inoculation of mice as described elsewhere (2,3). Swiss albino mice, Tumblebrook strain, and BALB/C mice, purchased from Jackson Memorial Laboratory, were used as test hosts for *Candida albicans* and *Streptococcus pyogenes* respectively. *Strain C58 mice and transplantable leukemia line I_b* originated with MacDowell and have been maintained in our laboratory since 1952. Young C58 mice moribund from growth of I_b cells served as donors of malignant leukocytes used for tests. Spleens harvested aseptically from leukemic mice were transferred to a sterile Petri dish and minced with Bard-Parker blades until particles were approximately 2 mm^3. Minced tissue flooded with Scherer's (4) maintenance solution (MS) was agitated gently and the supernatant fluid discarded. Tissue was minced further until particles approximated 1 mm^3, supernatant fluid transferred aseptically to a sterile tube, centrifuged to sediment gross particles, and supernate saved as the source of leukemic cells. White blood cells were separated from erythrocytes by cyclic differential centrifugation at low speed(5); line I_b leukocytes comprising the sedimented fraction were suspended in MS and counted in a hemocytometer. Per cent viability of cell suspensions was determined by incubation of an aliquot of cells with an equal volume of 0.2% trypan blue (dissolved in distilled water containing NaCl 0.8%, KCL 0.04%, glucose 0.1%); unstained cells were considered viable(6). Cell suspensions prepared as described above did not contain more than 5% dead cells.

Results. Accelerative effect of triiodothyronine on streptococcal infection of mice. The extraordinary sensitivity of male BALB/C mice to infection by *Streptococcus pyogenes*, type 18, provided an ideal experimental system to assess the effect of T3 on infection. Triiodothyronine, 0.1 ml (40 μg) was inoculated subcutaneously (SQ) into each of 6 test mice 24 hours before they were infected by intraperitoneal inoculation with streptococci as described previously(2). After inoculation, mice received 0.1 ml injections of T3 at 48 hour intervals during the 6-day observation period; control mice were inocu-

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