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COVALENT BINDING OF POLYAMINES TO PROTEINS IN HTC CELLS

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SUMMARY: When [^{3}H]-putrescine is incubated with HTC cells in the log growth phase, 12% of the total counts remain associated with the cells after repeated washings, and 0.15% of the cell-associated counts are bound to acid-precipitable material. After HCl hydrolysis, about half of the acid-precipitable radioactivity is in putrescine, with 25 to 35% of the remainder in spermidine plus spermine and less than 10% in a form less basic than putrescine. After pronase hydrolysis, 20-30% of the radioactivity resides in a fraction less basic than putrescine, which releases putrescine, spermidine and spermine subsequent to acid hydrolysis. The ability of acid hydrolysis to release polyamines that were not released by pronase suggests binding in isopeptide form similar to that formed by transglutaminase.

This report describes some aspects of the covalent binding of polyamines to cell associated proteins. Over the past twenty years evidence has accumulated that polyamines can be incorporated covalently into macromolecules (1-3). Data defining incorporation into proteins intrinsic to the cell (4,5) is less complete than that describing polyamine association with proteins found in body fluids. The following experiments were performed to obtain additional information about the nature of the polyamines covalently attached to cell-associated macromolecules, especially proteins, and to investigate the metabolic conversion of putrescine into spermidine and spermine in connection with this process.

HTC cells were maintained under standard conditions as reported earlier (6). Mid log phase cultures which had been fed 16 hours previously were used to measure incorporation of [^{3}H]-putrescine into cell protein. Putrescine (2,3[^{3}H] putrescine, (NEN) 100 uCi; 2.5 nmole) was added to 10 ml of cells (6.5 X 10^5/ml) and samples of 1.0 ml were taken at various times. The cells

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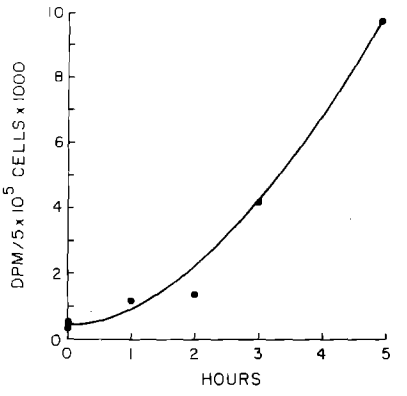

Figure 1. Time course of incorporation of [^{3}H] -putrescine into acid-precipitable macromolecular form by HTC cells in culture.

were washed repeatedly with phosphate buffered saline (NaCl 8.0 g/l; KCl 0.2 g/l; Na_2HPO_4 1.15 g/l; KH_2PO_4 0.2 g/l; pH 7.5) until the counts in the washes were at background level, and then similarly with 5% trichloroacetic acid (TCA). After solubilization, the final pellet was counted with ACS scintillation fluid in a Beckman model 7000 liquid scintillation counter. A typical experiment describing the time course of incorporation is shown in Figure 1.

After 5 hours of exposure to [^{3}H]-putrescine approximately 12% of the radioactivity had become associated with the cells. Acid-precipitable material generally represented about 0.15% of the total cell-associated counts. The acid-precipitable material was analyzed either after acid hydrolysis or after enzymatic hydrolysis which was followed by HPLC resolution of the components and then subsequent acid hydrolysis of the radioactive peaks. In each case acid hydrolysis of the material was carried out with 8.3 M HCl at 110° C for 16 hours in a sealed microtube in the presence of 6 nmol of carrier putrescine. The HCl was removed by lyophylization and the residue was dissolved in distilled water. The final sample was resolved into its different radioactive components by HPLC using the acetate buffer system previously described (7). Generally samples corresponding to 5 X 10^6 cells

TABLE 1

CONCENTRATIONS OF FREE POLYAMINES, mM

	Putrescine	Spermidine	Spermine
Growth Medium	0.003	0.017	0.127
Medium after incubation with cells	0.002	0.021	0.146
Intracellular	1.35	2.20	1.50

The concentration of polyamines was measured by their fluorescence in the o-phthalaldehyde reaction after separation by HPLC (7). Standards were run immediately before and after each run and concentrations were calculated from the peak heights.

The media were precipitated with 5% trichloroacetic acid before the supernatant fluid was analyzed. Cells from an 18 hour culture were frozen and thawed 5 times, then precipitated with 5% TCA and an aliquot of the supernatant fluid from 6 X 10^5 cells was applied to the column.

were hydrolyzed in a 200 µl volume. Pronase (Calbiochem) digestion was carried out using the method described by Arima et al., with the exception that hydrolysis was carried out in 0.1 M ammonium formate buffer, pH 6.5-7.0 (8). Enzymatic hydrolysis was followed by TCA precipitation of protein in the sample prior to HPLC resolution using the ammonium formate system (7). The ammonium formate in the fractions was removed by lyophylization prior to acid hydrolysis of the individual fraction.

Table 1 shows that the cellular concentrations of non-covalently bound polyamines are much higher than those existing in the culture medium. This implies that putrescine was taken up against a thousand-fold gradient, although it cannot be stated at present if the uptake involved active transport into the cells. Further studies are under way to elucidate the mechanism of this accumulation.

Analysis of the acid precipitable material from the HTC cells which establishes both the presence of polyamines in this fraction and the qualitative nature of this association is recorded in Table 2. This summary presents the quantitative data derived from the two hydrolytic approaches. Thus, HPLC resolution of the acid hydrolyzed material identifies the

TABLE 2

LABELED POLYAMINES COVALENTLY BOUND TO PROTEINS

Method of Hydrolysis	total cpm	Front# cpm	%	Putrescine cpm	%	Spermidine cpm	%	Spermine cpm	%
				EXPERIMENT 1					
Acid	3,382	278	8	1,539	46	1,260	27	305	9
Pronase	11,348	3,038	27	6,304	56	1,141	10	865	8
				EXPERIMENT 2					
Acid	8,206	197	2	6,221	76	1,566	19	222	3
Pronase	3,396	676	20	2,483	73	237	7	0	0

HYDROCHLORIC ACID HYDROLYSIS OF PRONASE PEAKS*

Source of Putrescine:	Pre-putrescine	Putrescine	Post-putrescine
Experiment 1	7%	77%	17%
Experiment 2	9	84	6

Source of Spermidine:	Pre-spermidine	Spermidine	Post-spermidine
Experiment 1	23	66	11
Experiment 2	77	0	22

Source of Spermine:	Pre-spermine	Spermine	Post-spermine
Experiment 1	43	31	25
Experiment 2	40	60	0

"Front" indicates all counts eluting prior to putrescine. This area includes, among others, γ-aminobutyric acid, γ-glutamyl putrescine, acetylated and hydroxylated putrescine.

* For details of procedure see text.

individual polyamines. In addition HPLC resolution of the products of enzymatic hydrolysis and subsequent acid hydrolysis of the individual peaks further permits the identification of the individual polyamine(s) in each peak. The observation that the polyamines are released by a proteolytic enzyme strongly supports the interpretation that the polyamines are bound to

protein rather than other acid-precipitable macromolecules. Therefore, a comparison between the findings from acid hydrolysis and enzymatic degradation provides a measure of the conjugation of polyamines in covalent links which are distinct from the conventional peptide bond.

Summation of the amount of the given polyamine in each individual peak after enzymatic degradation corresponds to the amount of the particular polyamine measured after acid hydrolysis of the total cell protein. Thus:

1. Pronase hydrolyzes the protein into acid soluble components which may be resolved with HPLC (3% remains insoluble in TCA) and
2. There is no significant degradation of isotopic protein in the absence of the proteolytic enzyme (less than 1%).

The major form of radioactivity derived from administered putrescine is identified as putrescine itself, of which at least 10% resides in proteolytic fragments which seem to be less basic than putrescine but which, upon acid hydrolysis, yield putrescine. Similarly, there is an approximate 2.5 fold increase in free spermidine after acid hydrolysis of the pronase-hydrolyzed peaks. The observation that there is material which elutes early in the HPLC system and whose position persists unchanged through both acid and enzymatic hydrolysis suggests the presence of material which is a metabolic derivative of the parent polyamine. Not more than 2% of this front-running material is $^{3}H_2O$.

DISCUSSION

It is well established that putrescine is readily metabolized to spermidine and spermine by cells (9,10), a finding which we corroborate. In addition to this conversion, putrescine and spermidine also appear to be incorporated into proteins by a covalent link other than the peptide bond. Although our data do not prove that this linkage occurs through the mediation

of transglutaminase, they are consistent with this possibility because a considerable part of the radioactivity after enzymatic hydrolysis appears in the HPLC chromatogram in a location consistent with γ-glutamylputrescine or di-γ-glutamylputrescine. Additional products may include oxidized derivatives, or acetylated or otherwise conjugated compounds which would release the parent polyamine after acid hydrolysis. These might include γ-aminobutyric acid, the isomers of γ-glutamylspermidine, putreanine, isoputreanine, hypusine or spermic acid. All of these polyamine derivatives have been identified both in body fluids and in tissue extracts(11,12).

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