EFFECT OF PRECIPITATING REAGENT UPON AMINO ACID INCORPORATION BY IN VITRO MAMMALIAN SYSTEMS

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Most investigations on the in vitro synthesis of proteins are based upon precipitability in trichloroacetic acid. Observations from this and other laboratories (Gardner et al., 1962 and Griffin et al., 1963) have indicated the apparent amino acid incorporation or protein biosynthesis may increase with more efficient precipitating reagents. A large increase in the incorporation of several amino acids in an in vitro tumor system was recorded when a trichloroacetic acid-tungstate (TCA-Tung) mixture replaced the usual TCA reagent (Griffin et al., 1963). Since different precipitating reagents may influence the outcome of studies involving messenger RNA, ribosomal organization, inhibitors, release of peptides, etc., the current study was initiated.

Experimental and Results. Ribosomal and amino acid activating fractions were prepared from Novikoff ascites tumor cells and rat liver as previously described (Griffin and O'Neal, 1962; Griffin et al., 1963; O'Neal and Griffin, 1963). Precipitating reagents employed in this study include: 5% TCA, 20% TCA and 0.25% Na tungstate in 5% TCA (Gardner et al., 1962). The last reagent forms a voluminous precipitate. In this study the tungstate was dissolved in a small quantity of water.

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TABLE I

Role of Precipitating Reagent Upon Apparent Amino Acid Incorporation by Tumor and Liver Systems

μμ moles amino acid per mg. ribosomal protein

Amino Acids	Tumor System		Liver System	
	TCA	TCA-Tungstate	TCA	TCA-Tungstate
Alanine	113	161	58	104
Arginine	116	227	119	167
Aspartic Acid	41	87	16	46
Glutamic Acid	67	118	19	27
Glycine	64	131	12	28
Histidine	28	79	16	47
Isoleucine	57	96	25	49
Leucine	110	163	44	103
Lysine	217	455	73	209
Methionine	38	67	22	40
Phenylalanine	31	49	21	43
Proline	83	167	33	51
Serine	80	141	29	80
Threonine	78	137	32	83
Tyrosine	62	115	49	61
Valine	62	131	15	63

The complete incubation system includes: 0.1 ml ribosomes (1 mg protein); 0.2 ml S100pH5•2X (2 mg protein); .005 μ mole C^{14} amino acid; .005 μ mole each of cold amino acids; 0.25 μ mole ATP; 7.5×10^{-3} μ mole GTP; 2.5 μ mole phosphoenolpyruvate; 5.0 μg phosphoenolpyruvate kinase; 3 μ mole $MgCl_2$. Buffer was added to a total volume of 0.5 ml; the reaction mixtures were incubated at 37° C. for 40 minutes. Samples of .05 ml were pipetted onto Whatman #3MM paper discs. The discs were twice extracted with cold precipitating reagent and hydrolyzed for 15 minutes in the reagent at 90° C. The last procedure was repeated and the discs washed once with cold reagent and twice in ethanol. The discs were dried and counted in a scintillation counter (counting efficiency 45-50%).

This solution was added to 5% TCA and the mixture allowed to stand until the precipitate had formed and settled. The clear supernatant fraction was used as the precipitating reagent.

Substitution of TCA-Tung for TCA resulted in a doubling of the incorporation of several amino acids in the ribosomal systems of the tumor and liver (Table I). In Table II the relative values obtained by treatment with 5% TCA, 20% TCA and the TCA-Tung are shown. The TCA-Tung treatment again resulted in an increase in incorporation over that observed with 5% TCA, while the 20% TCA gave even lower incorporation values. Addition of puromycin resulted in a 30% decrease in the incorporation of arginine and an 80% decrease for lysine, serine and valine when 5% TCA was used. With TCA-Tung the decrease in arginine incorporation was 25% and the decrease for the other amino acids was 50%.

TABLE II

Comparison of Precipitating Reagents Upon Amino Acid Incorporation by Ribosomal System of Tumor in Absence or Presence of Puromycin

Amino Acids	5% TCA		20% TCA		TCA-Tungstate	
		+Puromycin*		+Puromycin		+Puromycin
Arginine	101	72	71	56	190	149
Lysine	120	20	76	23	261	127
Serine	52	10	38	7	102	44
Valine	39	3	27	3	86	46

μμ moles amino acid incorporated per mg ribosomal protein.
*Puromycin, 0.2 μ mole added per incubation tube.

The increased amino acid incorporation obtained when TCA-Tung was used may be attributed to more efficient precipitation of proteins or peptides or possibly to the failure of this reagent to hydrolyze the amino acyl-RNA complex. Formation of this complex was achieved by incubation of

TABLE III

Determination of Amino Acid Activation Employing Different Precipitating Reagents

Amino Acid	TCA		TCA-Tungstate	
	Cold	Hot	Cold	Hot
Arginine	2500	770	2510	1390
Glutamic Acid	3800	505	3890	1100
Lysine	4650	200	4190	1310
Valine	4260	67	3980	920

See legend, Table I. S100,pH5•2X prepared from tumor was employed and values are expressed in total counts per incubation tube.

the activating fraction in the system minus ribosomes. It may be observed that the extent of activation for each amino acid was approximately the same following extraction with either cold TCA or TCA-Tung (Table III). Extraction with hot TCA reduced the counts to near background as expected. However, arginine and glutamic acid were still measureable. Considerable activity remained on the paper discs for many amino acids following extraction with hot TCA-Tung. These results indicate that there may be some apparent incorporation taking place in the non-ribosomal system. In order to explore this further, incubated samples were adjusted to pH 11.0 and reincubated at 37° C. to hydrolyze the aminoacyl-RNA. Treatment with hot TCA-Tung still indicated a higher incorporation than was obtained for TCA (Table IV); however, this differential was not as large as that obtained without the alkaline treatment (Table I). Certain labeled amino acids (arginine and glutamic acid) were also present on the paper discs following extraction with hot TCA-Tung and to a lesser extent with hot TCA, even when ribosomes were omitted from the incubation mixture (Table IV).

TABLE IV

Effect of Treatment at pH 11.0 on Apparent Amino Acid Incorporation

Amino Acids	Complete System		Complete System - Ribosomes	
	TCA	TCA-Tungstate	TCA	TCA-Tungstate
Alanine	1580	1880	30	30
Arginine	1120	1740	550	850
Glutamic Acid	2540	3040	670	860
Leucine	810	950	30	40
Lysine	1860	2260	100	140
Phenylalanine	460	460	20	10
Proline	1820	2420	140	220
Valine	1700	2160	70	220

Same procedure as described in legend to Table I. Following usual incubation, the pH of each tube was adjusted to 11.0 by addition of $NaHCO_3$ and the tubes reincubated for 15 min. at 37°. 0.05 ml samples were removed and treated as previously described. Values are expressed in total counts per incubation tube.

Discussion. These findings would indicate that the results and subsequent interpretation of *in vitro* amino acid incorporation studies may be influenced considerably by the precipitating reagent employed. Large increases in the apparent incorporation of several amino acids were evident when TCA-Tung was used in place of TCA. Gardner *et al.* (1962), using more efficient precipitating reagents, detected polypeptides that were not precipitated by TCA. This enabled them to extend the code designations. In agreement with others we noted that addition of puromycin to the incubation mixtures greatly reduced the incorporation of most amino acids when TCA was employed as the precipitating reagent. With TCA-Tung, however, this inhibitory effect was less pronounced indicating a greater apparent incorporation. Presumably, the TCA-Tung will precipitate puromycin-containing peptides (Nathans *et al.*, 1962) which are not detected by the TCA.

Labeled arginine, glutamic acid, lysine, and valine were present in the incubated, ribosomal-free activating fraction of tumor, and to a lesser extent, in the activating fraction of liver following extraction with hot TCA-Tung. The possibility exists that this reagent does not hydrolyze the amino-acyl-RNA; however, treatment of S-RNA-C^{14} arginine or valine with hot TCA-Tung or incubation at pH 11.0 resulted in complete hydrolysis. It would appear that the S100,pH5 fraction contains ribonucleoproteins capable of synthesis of peptides or that certain amino acids undergo end-group additions to peptides that are precipitated by the TCA-Tung. The enhanced incorporation by the non-ribosomal system is energy dependent and does not occur following heating of the S100,pH5 fraction to 70° C. Incorporation is not dependent upon GTP, is destroyed by addition of ribonuclease but is only slightly inhibited by puromycin. This apparent incorporation by the activating fraction may be related to the soluble amino acid-incorporating system found in rat liver by Kaji et al. (1963). We are attempting to characterize the peptides synthesized by the mammalian system and to obtain a better understanding of the mechanism of action and the limitations of the reagents that play an important role in defining amino acid incorporation and protein biosynthesis.

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