FACTORS AFFECTING PROTEIN SYNTHESIS IN VITRO IN RABBIT RETICULOCYTES*

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Rabbit reticulocytes *in vitro* rapidly incorporate labeled amino acids into their proteins. The process is accelerated by the plasma of every mammal investigated and also by extracts of normal erythrocytes, rabbit reticulocytes, liver, spleen, and yeast (1). We have described two sets of stimulating factors: one of these sets consists of certain amino acids (1), the other of fructose-amino acids in liver (2–4). The latter set is ineffective without the addition of iron to the reaction medium. The effect of iron has been referred to in preceding publications (2–5), but without detail. After the necessity of adding iron was recognized, in order to obtain a maximal rate of protein synthesis the reaction mixture was improved further by adding to it certain substances which depend upon added iron for their effect. These increased the effect of plasma. Eventually the total (potential as well as actual) accelerating effects of plasma and liver extract were accounted for by known substances. This led to the devising of a reaction mixture formula in which the amino acid incorporation is about five times as fast as that observed when the cells are incubated in saline.

Procedure

In all of these experiments the labeled amino acid was carboxyl-C^{14}-L-leucine, which had been synthesized as previously described (6). Comparable results were obtained in many other experiments with labeled glycine, histidine, and lysine (1, 5).

Except that calcium and phosphate salts were omitted from the saline solution (see below), the experimental procedure throughout, from the production of the reticulocytosis to the measurement of the radioactivity

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of the heme-free protein, was as previously described (1, 5). The incubation time was 4 hours, unless stated otherwise. In these experiments the hemoglobin was not separated from the other proteins. The hemoglobin, as measured by the method of Drabkin and Austin (7), in the reticulocytes as prepared by us is about 15 per cent and constitutes nearly all of the dry weight of the material precipitated by trichloroacetic acid. These facts and the results of experiments in which the hemoglobin was isolated (5) warrant referring to amino acid incorporation into the proteins of rabbit reticulocytes as protein or hemoglobin synthesis.

Results

Table I is a summary of the main factors we have found which accelerate protein synthesis *in vitro* in rabbit reticulocytes. Not included are fructose-amino acids whose effects have been described (2–5) and are referred to below in discussion of the effect of iron. It is seen that iron and glucose, separately or together, had little accelerating effect. The amino acid mixture alone caused an increase of 70 per cent. Amino acids and iron, but not amino acids and glucose, acted synergistically; glucose acted synergistically when added with amino acids and iron. Transferrin, added with amino acids, iron, and glucose, increased the rate still further. These results indicate that for a high rate of synthesis the constituents of hemoglobin, *i.e.* amino acids and iron, and an energy source such as glucose must be available in adequate amounts. The result with transferrin points to the usefulness of an iron-chelating agent even though it may not be strictly necessary.

Amino Acid Mixture—The amino acid mixture used had the following composition expressed as micrograms per ml. of reaction mixture: L-alanine 45, L-arginine 21, L-aspartic acid 95, L-cysteine 12.5, glycine 100, L-glutamine 70, L-histidine 90, L-hydroxyproline 37.5, L-isoleucine 10, L-lysine 65, L-methionine 12.5, L-phenylalanine 65, L-proline 40, DL-serine 90, L-threonine 50, L-tryptophan 15, L-tyrosine 37.5, and L-valine 90. L-Leucine was provided in the carboxyl-C^{14} form and at a concentration of 10^{-3} M (131 γ per ml.).

Only nine of the above nineteen amino acids were found to be in any degree limiting during a 4 hour experiment. These, with the exception of leucine, are given in Table II. In other experiments (1) it had been found that leucine is severely limiting.

Three points may be made regarding the results such as those in Table II. Amino acids vary in the degree to which they are limiting, histidine being the most limiting. In a reaction mixture from which one of the limiting amino acids is withheld, the rate is at a characteristic suboptimal level from the beginning and persists at this level. Throughout the 4 hours

glutamine had an accelerating effect, although added glutamic acid had no effect. In separate experiments it was found that C^{14}-labeled glutamic

TABLE I

Factors Accelerating Protein Synthesis in Rabbit Reticulocytes in Vitro

Amino acid mixture	Iron, 5 γ per ml.	Glucose, 1 mg. per ml.	Transferrin, 50 γ per ml.	Rate of protein synthesis
−	−	−	−	100
−	+	−	−	100
−	−	+	−	110
−	+	+	−	111
+	−	−	−	170
+	+	−	−	351
+	−	+	−	170
+	+	+	−	497
+	+	+	+	597

The composition of the amino acid mixture is given in the text. The iron was in the form of ferrous ammonium sulfate. The transferrin was a crystalline metal-free preparation.

TABLE II

Effect of Amino Acid Composition

The results are expressed as per cent of that in saline alone without added amino acids. The complete reaction mixture contained the amino acid mixture described in the text, 5 γ per ml. of iron as ferrous ammonium sulfate, and 1 mg. per ml. of glucose. It did not contain transferrin.

Amino acid composition	1 hr.	2 hrs.	3 hrs.	4 hrs.
Complete	447	450	489	475
" without histidine	94	87	94	96
" " valine	185	170	181	187
" " phenylalanine	176	183	181	200
" " serine	215	227	220	228
" " lysine	235	229	237	222
" " tryptophan	257	267	264	285
" " tyrosine	262	280	295	297
" " glutamine	304	339	353	366

acid was not incorporated at all, whereas C^{14}-glutamine was extensively incorporated. It was surprising that glutamic acid was not incorporated. A low rate of incorporation of labeled glutamic acid might have been explained by extensive dilution of the labeled amino acid by the glutamic acid synthesized within the cell, but there was no incorporation whatsoever. In view of the rapid incorporation of glutamic acid into

other cells which has been observed, a likely explanation would appear to be that glutamic acid is unable to penetrate into the reticulocyte, whereas glutamine can. It has been observed that glutamic acid penetrates the cells of liver slices slowly (8).[1]

The limiting amino acids in rabbit reticulocytes include those which are both dispensable and indispensable. It is a reasonable surmise that the reason that methionine and threonine (amino acids which are constituents of hemoglobin and indispensable in the diet of the animal) are not limiting is that they are present in the pool of amino acids within the reticulocytes in amounts sufficient for the protein synthesis that occurs in the 4 hours incubation *in vitro*. On the other hand, although reticulocytes synthesize serine from glycine (1), and presumably also synthesize glutamic acid, evidently they do not make enough for a maximal rate of protein synthesis. These findings illustrate that the amino acid requirements of different tissues may be quite different from those of the animal as a whole.

TABLE III

Effect of Partial Histidine Deficiency

The results are expressed as per cent of that in the complete reaction mixture.

Amount of histidine added to otherwise complete reaction mixture		Rate in successive hrs.			
		0–1	1–2	2–3	3–4
γ per ml.	*M*				
0	0	23	18	27	23
9.25	0.575×10^{-4}	81	30	33	24
18.5	1.15×10^{-4}	104	77	39	25
27.75	1.72×10^{-4}	100	98	93	47
37.0	2.30×10^{-4}	100	98	88	80
46.25	2.87×10^{-4}	100	97	98	102
74.0	5.75×10^{-4}	100	100	100	100

Table III shows in more detail how the quality of the amino acid mixture may determine the rate of protein synthesis. With suboptimal concentrations of a limiting amino acid, in this case histidine, the rate is at first nearly maximal and then declines, but may remain well above the minimal rate. Similar results were obtained with phenylalanine and valine. The

[1] We are indebted to Dr. Eugene Roberts (City of Hope Medical Center, Duarte, California) for the demonstration within washed reticulocytes of relatively large amounts of both glutamic acid and glutamine. Roberts and Tanaka (12) had found that ascites tumor cells possess only a limited permeability to glutamic acid and are quite permeable to glutamine. Reticulocytes, evidently, resemble ascites tumor cells in this respect.

findings are analogous to those in growing animals maintained on a quantitatively suboptimal protein ration.

Iron—The effect of the addition of iron to the reaction mixture is shown in Table IV. $FeSO_4(NH_4)_2 \cdot SO_4 \cdot 6H_2O$ was used for the most part, ferrous chloride was equally effective but less convenient, and ferric chloride was almost as effective. To obtain consistent and maximal effects, it was necessary to add the iron to the reaction mixture after the reticulocytes as the last ingredient before incubation. The reaction mixture was somewhat alkaline (about pH 8) before the reticulocytes were added and before they were placed under 95 per cent oxygen and 5 per cent carbon dioxide. The iron may be precipitated when added at this point.

TABLE IV
Effect of Iron

Reaction mixture except for iron and transferrin	Concentration of iron	Leucine incorporated
	$M \times 10^{-5}$	*μmoles per gm. protein*
Saline alone	0	3.9
Complete	0	6.8
"	0.35	9.3
"	0.89	12.1
"	1.79 (1 γ per ml.)	17.7
"	3.58	19.1
"	8.95	19.6
"	17.90	19.6

With enough iron added to an otherwise complete reaction mixture, about three times as much leucine was incorporated in 4 hours. If one assumes that all the leucine incorporated represents newly synthesized hemoglobin (a warrantable assumption), data such as those in Table IV indicate that about three times as much iron needed to be added to the reaction mixture as was incorporated into hemoglobin. The calculation is as follows: hemoglobin contains 75 residues of leucine per 4 atoms of iron (9, 10). Each beaker contained 0.5 ml. of packed cells in 4 ml. of reaction mixture, and therefore about 75 mg. of protein, most of which was hemoglobin. An increased incorporation of 12 μmoles of leucine per gm. of protein corresponds per beaker to an increased incorporation of about 0.5×10^{-1} μmole of iron into hemoglobin. To obtain this increase the addition of 1.6×10^{-1} μmole of iron per beaker was needed.

Evidently there is very little iron in reticulocytes available for hemoglobin synthesis. The data indicate that some of the iron added to the reaction medium was rendered unavailable. This is the interpretation we have placed on data such as those in Table V. It is seen that during

the 1st hour the addition of a very small amount of iron, between 0.017 and 0.044 μmole, had a nearly maximal effect, whereas, over a 4 hour

TABLE V

Increase in Iron Bound As Hemoglobin with Increasing Amounts of Iron Added to Reaction Medium

Iron added to reaction medium		Total iron bound as hemoglobin during			Increase in bound iron after addition of iron
Concentration	Total	1 hr.	2 hrs.	4 hrs.	
γ per ml.	*μmoles × 10*	*μmole × 10*	*μmole × 10*	*μmole × 10*	*μmole × 10*
0	0	0.092	0.14	0.16	
1.0	0.17	0.16	0.21	0.24	0.08
2.5	0.44	0.17	0.25	0.30	0.14
5.0	0.89	0.18	0.28	0.34	0.18
10.0	1.79	0.17	0.30	0.38	0.22
20.0	3.58	0.18	0.30	0.38	0.22
40.0	7.16	0.18	0.30	0.38	0.22

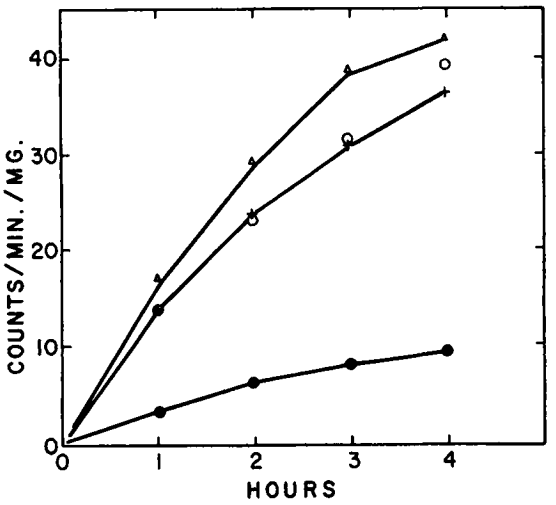

FIG. 1. The symbols used are △, a mixture of plasma + amino acids + iron + glucose; ○, a mixture of non-protein plasma filtrate dialyzed + amino acids + iron; +, a mixture of amino acids + iron + glucose; ●, blank.

period, between 0.089 and 0.179 μmole was required for a maximal increase in hemoglobin iron of only 0.022 μmole.

The addition of cobalt (as $CoCl_2$) also accelerated protein synthesis in reticulocytes, but less than iron. Even at 10^{-4} M concentration cobalt effected only a 50 per cent increase in leucine incorporation, as compared

with 130 per cent increase by the same concentration of iron. When cobalt was added with iron at comparable concentrations, only the accelerating effect of the iron was obtained.

Experiments were carried out also with aluminum, manganese, molybdenum, and zinc at concentrations of 0.2 to 1.0 $\times 10^{-4}$ M with and without added iron. They were all slightly inhibitory when the iron was 10^{-4} M.

TABLE VI

Comparison of Accelerating Effects of Plasma (Rabbit) and of Transferrin

The results are expressed as per cent of value in an otherwise complete reaction mixture.

Protein added	Amount added per ml. reaction mixture	Transferrin concentration added to reaction mixture		Incorporation of leucine
	ml.	*γ per ml.*		
None		0		100
Plasma	0.05	125	Estimated (11)	141
"	0.025	62.5	"	135
"	0.005	12.5	"	125
"	0.001	2.5	"	118
"	0.0005	1.25	"	100
Transferrin		200		141
"		60		134
"		20		132
"		4		110
"		2		100
	γ			
Rabbit serum albumin	500	0		97
Bovine " "	500	0		98
Human " "	500	0		97

At lower concentrations of iron they had no effect. No transferrin was used in any of the above experiments with metals.

Transferrin causes an increase above that obtained in a reaction mixture optimal with respect to amino acids, glucose, and iron. A similar effect had been obtained with whole plasma (rabbit and human) (Fig. 1); in twenty-three experiments rabbit plasma caused an increase of 25 ± 12 per cent over that in an otherwise optimal reaction mixture. The active principle was not dialyzable and disappeared upon boiling. Fractionation suggested that it might be transferrin. As Table VI shows, this surmise appears to have been correct. The estimate in Table VI of the transferrin in the plasma added is based on Drabkin's value of 0.25 gm. per 100 ml. of plasma (11). The transferrin used was kindly provided by Dr. J. L.

Oncley of Harvard University.[2] The specificity of transferrin is attested to further by the absence of any effect of rabbit, bovine, or human serum albumin.

Presumably the effectiveness of transferrin comes from its capacity to chelate and thus transfer iron. It is surmised that the accelerating effects of fructose-amino acids and citrate are for the same reason (2–5); the maximal accelerating effect is obtained with about 10^{-6} M transferrin and 5×10^{-4} M fructose-amino acids or citrate. At suboptimal concentrations of added iron, $<10^{-5}$ M, fructose-amino acids or citrate is more effective than transferrin; at optimal concentrations of iron, 4×10^{-5} M, or higher, transferrin is more effective.

TABLE VII

Effect of Glucose on Incorporation of Leucine into Reticulocyte Proteins

	Incorporation, c.p.m. mg.				Effect of glucose on incorporation, c.p.m. mg.			
	1 hr.	2 hrs.	3 hrs.	4 hrs.	1 hr.	2 hrs.	3 hrs.	4 hrs.
Saline	3.32	4.75	6.38	7.43				
With glucose	3.12	5.03	6.89	7.72	−0.20	0.38	0.51	0.29
" amino acids	14.59	22.94	25.78	26.01				
" glucose + amino acids	15.01	26.15	34.05	36.84	0.42	3.21	8.27	10.83
Percentage increase of incorporation effected by glucose					2.7	12.2	24.2	29.3

The added glucose was 1 mg. per ml. The amino acids were the complete mixture described above, and the iron was 5 γ per ml.

Table VII shows the accelerating effect of glucose added to the reaction mixture. It is seen that the effect was little in the 1st hour of incubation and became progressively greater, presumably as the carbohydrate initially in the reticulocytes was consumed.

Tables VIII, IX, and X show some results of varying the electrolyte composition of the saline in which the cells were incubated. There was much less incorporation in an all potassium solution. An all sodium or a half sodium half potassium afforded the maximal rate (Table VIII). Phosphate was inhibitory, and its inhibitory effect was neutralized by magnesium (Table IX). Calcium was inhibitory, and its inhibitory effect was relieved by citrate (Table X). After these observations the Krebs-Henseleit solution we had used heretofore was modified by omission of the calcium and phosphate salts.

[2] It was prepared from human plasma, crystalline, metal-free, and consisted of 96 per cent β_1 metal-binding protein, 3 per cent β-2 and γ- and 1 per cent of α-globulins.

TABLE VIII

Effects of Sodium and of Potassium

Nature of saline solution	Leucine incorporated
	μmoles per gm. protein
Krebs-Henseleit saline solution	20.1
All sodium " "	19.8
" potassium " "	10.9
Half sodium + half potassium saline solution	19.0

"All sodium" and "all potassium" saline solutions: the potassium and sodium salts of the Krebs-Henseleit solution were replaced by the corresponding sodium and potassium salts, respectively.

TABLE IX

Effects of Magnesium and of Phosphate

Mg	PO_4	Leucine incorporated
M	*M*	*μmoles per gm. protein*
0	0	18.2
0.008	0	18.6
0.0016	0	18.6
0	0.008	11.0
0	0.0016	17.6
0.008	0.008	16.8
0.008	0.0016	18.6
0.0016	0.008	15.2
0.0016	0.0016	17.4

The basic saline solution was the Krebs-Henseleit mixture from which the calcium magnesium and phosphate salts were omitted.

TABLE X

Effects of Calcium and of Citrate

$CaCl_2$	Citrate	Leucine incorporated
M	*M*	*moles per gm. protein*
0	0	18.4
2.8×10^{-3}	0	11.6
0	0.25×10^{-3}	19.4
0	1.0×10^{-3}	19.1
2.8×10^{-3}	0.25×10^{-3}	17.3
2.8×10^{-3}	1.0×10^{-3}	19.7

The basic saline solution was as in Table XIII.

TABLE XI

Effect of Some Inhibitors

The results are expressed as per cent inhibition.

	Molar concentration			
	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Arsenate	90	35	4	0
Arsenite	99	5	2	1
2,4-Dinitrophenol	97	96	24	3
N-Ethylmaleimide	6	0	0	0
Diisopropylfluorophosphate	0	0	0	0
p-Cl Hg-benzoate	95	9	0	0
Hg-phenylsulfonate	95	21	2	0
Lead acetate	98	96	79	18

TABLE XII

Effect of Some Inhibitory Metals

The results are expressed as per cent inhibition.

	Molar concentration			
	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Ammonium aluminum sulfate	17	2	0	0
Potassium aluminum sulfate	10	4	4	0
Antimony potassium tartrate	98	93	5	0
Cupric chloride	16	0	0	0
Gold chloride	96	84	2	0
Lead acetate	98	96	79	18
Mercuric acetate	100	92	5	0

TABLE XIII

Effects of Some Amino Acid, Purine and Pyrimidine Analogues, and Antibiotics

The results are expressed as per cent inhibition.

	Molar concentration				
	5×10^{-3}	1×10^{-3}	5×10^{-4}	2.5×10^{-4}	1×10^{-4}
Benzimidazole	19	2	0	0	0
2,6-Diaminopurine sulfate	18	13	0	0	0
8-Azaguanine	12	0	0	0	0
4-Phthalimido-2,6-dimethylpyrimidine	42	0	0	0	0
Chloramphenicol	93	13	6	0	0
Aureomycin	71	56	45	12	0

Tables XI, XII, and XIII show the effects of a variety of inhibitors at a range of concentrations. The most unexpected result was that lead (as lead acetate) was the most powerful inhibitor found; it was much more inhibitory than antimony, gold, or mercury, which last were about the same. Among the antibiotics tested, Aureomycin was the most inhibitory and chloramphenicol next; bacitracin, penicillin G, and streptomycin caused less than 10 per cent inhibition at 5×10^{-3} M. The following amino acid, purine and pyrimidine analogues, caused less than 10 per cent inhibition at 5×10^{-3} M: *o*-fluorophenylalanine, 3-fluoro-L-tyrosine, β-2-thienylalanine, 4-amino-5-imidazolecarboxamide, isoguanine sulfate, 6-mercaptopurine, 6-aminouracil, 5-bromouracil, 4,6-dihydroxypyrimidine, and 2-thiocytosine.

DISCUSSION

The effects of varying degrees of amino acid deficiency and of iron are analogous to nutritional experiments in animals. Even the extraordinary toxicity of lead might be cited as an analogue of lead poisoning *in vivo*.

These experiments show that different tissues have amino acid requirements which are quite different from the animal as a whole and that the amino acid requirement of the animal as a whole is not merely a weighted average of that of all the tissues individually. Amino acids made in one tissue in excess of need may be used by another tissue that does not synthesize optimal amounts, *e.g.* serine, tyrosine, and glutamine in reticulocytes.

SUMMARY

The effects of a variety of substances added to the reaction medium on the rate of protein synthesis in rabbit reticulocytes *in vitro* are described.

1. Protein synthesis was fastest when the amino acid and iron constituents of hemoglobin, which is the main protein, were present together with glucose.

2. The amino acid in which the reticulocytes were most deficient was histidine. When histidine was omitted from an otherwise optimal reaction mixture, the rate was a little less than in saline alone. Next in order of their deficiency were valine, phenylalanine, serine, lysine, tryptophan, tyrosine, and glutamic acid. No evidence was found of a deficiency of other amino acids.

3. Reticulocytes appeared to be impermeable to glutamic acid; however, glutamine was extensively incorporated presumably as glutamic acid.

4. Reticulocytes are deficient in and sensitive to added iron. The addition of as little as 3×10^{-6} M iron caused a 50 per cent increase in protein synthesis. Ferric was almost as effective as ferrous iron.

5. Some of the iron added to the reaction medium became unavailable.

Transferrin (siderophilin) in the reaction medium increased the availability of the iron for a longer time.

6. Cobaltous ions in the reaction medium have an accelerating effect, which is less than and not additive to that of iron.

7. Replacement of all the sodium in the saline solution by potassium had an inhibitory effect; replacement of half the potassium by sodium ions restored the maximal rate. Phosphate and calcium ions had inhibitory effects which were neutralized, respectively, by magnesium ions and citrate.

8. Among a variety of inhibitors lead was the most powerful. Aureomycin was the most inhibitory antibiotic tested. A number of amino acid, purine and pyrimidine analogues were tested. A few were slightly inhibitory at concentrations as low as 10^{-3} M, and the remainder were not inhibitory.

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