INCORPORATION IN VITRO OF LABELED AMINO ACIDS INTO PROTEINS OF RABBIT RETICULOCYTES*

By HENRY BORSOOK, CLARA L. DEASY, A. J. HAAGEN-SMIT, GEOFFREY KEIGHLEY, AND PETER H. LOWY

(*From the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California*)

(Received for publication, November 13, 1951)

Continuing our work on the incorporation of labeled amino acids into proteins (1), we have begun a study of the incorporation *in vitro* of C^{14}-labeled glycine, L-histidine, L-leucine, and L-lysine into the proteins of rabbit reticulocytes. In preliminary experiments the incorporation into the hemoglobin isolated from the reticulocytes was determined. But, after it was found that plasma contains factors accelerating amino acid incorporation, it was decided to proceed as rapidly as possible toward the identification of these factors; we have, therefore, measured incorporation into the total proteins of the reticulocytes, since isolation of the hemoglobin was time-consuming. The results obtained with hemoglobin and with the total proteins are essentially the same, indicating that the other proteins of the reticulocytes incorporate amino acids at approximately the same rate as hemoglobin.

Production of Reticulocytosis—Reticulocytosis was produced in adult rabbits by a modification of the method of London *et al.* (2). 1 ml. of a neutralized 2.5 per cent aqueous solution of phenylhydrazine hydrochloride was injected subcutaneously each day for a week; over 90 per cent of the circulating red cells were then reticulocytes. In some cases 0.05 mg. of folic acid and 0.05 γ of vitamin B_{12} were injected daily, in addition to the phenylhydrazine.[1]

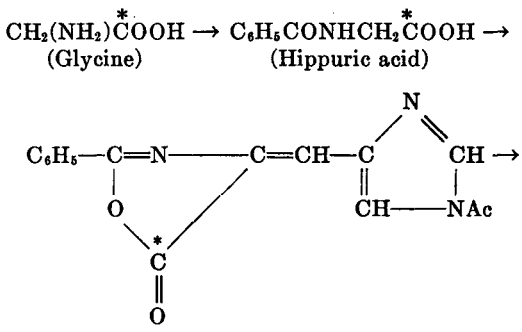
Labeled Amino Acids—The radioactive amino acids were labeled with C^{14}

* These studies were aided by a contract between the Office of Naval Research, United States Navy Department, and the California Institute of Technology, Division of Biology (NR-164-304). It was also supported (in part) by a research grant from the National Institutes of Health, United States Public Health Service, and by a grant from the American Cancer Society. The C^{14} used in this investigation was supplied by the Carbide and Carbon Chemicals Corporation, Oak Ridge, Tennessee, and obtained on allocation from the United States Atomic Energy Commission.

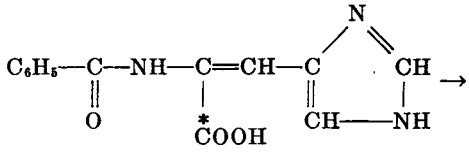
A preliminary report of this work was given at the meeting of the Federation of American Societies for Experimental Biology, April 29 to May 3, 1951 (*Federation Proc.*, **10**, 18 (1951)).

[1] The folic acid was obtained from the Nutritional Biochemicals Corporation and the vitamin B_{12} from Chas. Pfizer and Company, Inc.

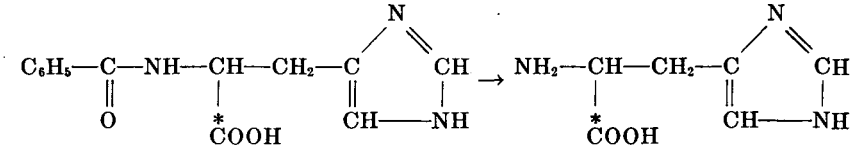
in the carboxyl group. Glycine was synthesized by the method of Sakami *et al.* (3); syntheses of L-leucine and L-lysine were described previously (1).



(2-Phenyl-4- (1-acetylimidazole-4 (or 5) -methylidene) oxazolone)



(α-Benzoylamino-β-imidazole-4 (or 5) -acrylic acid)



(Benzoyl-DL-histidine) DL-Histidine

Carboxyl-C^{14}-histidine had been prepared by Bouthillier and D'Iorio (4), but the yield, on the basis of $NaC^{14}N$ used, was only 6 per cent. A new synthesis giving a 20 per cent yield was therefore developed.

Hippuric Acid—To 2.11 gm. of glycine-carboxyl-C^{14} dissolved in 22 ml. of 10 per cent sodium hydroxide, a mixture of 9.65 ml. of benzoyl chloride and 45 ml. of 10 per cent sodium hydroxide was added gradually with stirring. Stirring was continued until all the benzoyl chloride was destroyed. Acidification precipitated hippuric acid, which was purified by repeated extraction with boiling ligroin. Yield, 4.96 gm. (98.5 per cent theory); m.p. 183–185° (uncorrected).

2-Phenyl-4-(1-acetylimidazole-4(or 5)-methylidene)oxazolone—4.96 gm. of hippuric acid were treated with 3.10 gm. of imidazoleformaldehyde (5–7) and with 3.10 gm. of sodium acetate in 12 ml. of acetic anhydride. Yield, 5.74 gm. (74 per cent theory).

α-Benzoylamino-β-imidazole-4(or 5)-acrylic Acid—11.95 gm. of oxazolone were boiled with sodium carbonate (5). Yield of hydrated acid, 9.81 gm. (84 per cent theory).

*Benzoyl-*DL*-histidine*—To 1.50 gm. of α-benzoylamino-β-imidazole-4(or 5)-acrylic acid suspended in 15 ml. of water 20 gm. of 2.15 per cent sodium amalgam were added gradually. The aqueous solution, separated from the mercury, was acidified to pH 5.2. The precipitate was filtered and washed with water; additional crystals were obtained from the concentrated mother liquors. Yield, 51 per cent theory; m.p. 238–239° (uncorrected).

DL-*Histidine Hydrochloride*—1.95 gm. of benzoyl-DL-histidine were refluxed for 4 hours with 100 ml. of 20 per cent hydrochloric acid. The aqueous phase was dried *in vacuo*, after the benzoic acid was removed by repeated ether extraction.

DL-*Histidine*—The free base was obtained either by treatment of the hydrochloride with silver carbonate in the usual way or by electrodialysis. The latter method gives a purer product and a greater yield. 170 mg. of hydrochloride dissolved in 100 ml. of water were placed in a glass cell, in which were suspended two cellophane bags 2.5 cm. wide, each containing a platinum wire mesh electrode and distilled water to the level of the surrounding solution. The electrodes were in series with a 30 watt, 115 volt lamp and connected to a 120 volt direct current. The solution was stirred occasionally with a glass rod; every 30 minutes the electrodialysis was interrupted and the catholyte and anolyte were removed by pipette and replaced by distilled water. After 4 hours the combined catholytes were evaporated to dryness *in vacuo*. Yield, 97 per cent theory.

Calculated, N 27.09; found, N 27.05[2]

L-*Histidine*—821 mg. of DL-histidine dissolved in 3.75 ml. of water were heated to a clear solution with 800 mg. of L-tartaric acid, prepared by racemization of DL-tartaric acid (8) and resolution with cinchonine (9, 10). A precipitate was obtained from the cooled solution on standing, after seeding with a crystal of L-histidine-L-tartrate. Yield, 671 mg. (83 per cent theory); $[\alpha]_D^{26} = -12.2°$.

Calculated, C 39.34, H 4.96, N 13.77; found, C 39.48, H 5.17, N 13.67

The free base was prepared from the tartrate by the electrodialysis procedure previously described.

EXPERIMENTAL

Procedure—The reaction mixtures were incubated in 20 ml. beakers at 37.5° with rocking in the Dubnoff apparatus (11) under 95 per cent oxygen

[2] Microanalyses by Glenn A. Swinehart.

and 5 per cent carbon dioxide; in a few anaerobic experiments a mixture of 95 per cent nitrogen and 5 per cent carbon dioxide was used. A few experiments incubated at 36° and at 40° gave incorporations 90 per cent and 120 per cent, respectively, of that at 37.5°.

The total volume in each reaction beaker was 4 ml., of which 3.5 ml. were the usual Krebs-Henseleit solution (12), plasma, or extracts, with additions as indicated, and 0.5 ml. was washed cells. The cells were separated from the plasma of the heparinized blood, obtained by heart puncture or from the ear of the anemic rabbits, by centrifugation in a refrigerated centrifuge. The cells were then washed twice with 8 volumes of a Krebs-Henseleit solution which contained no sodium bicarbonate and was 1.1 times as strong in the other salts as the usual Krebs-Henseleit solution.

Each beaker contained 0.6 mg. of heparin sodium.[3] The concentration of each labeled amino acid was 0.001 M; the specific activity of the glycine was either 15,500 or 31,000 c.p.m. per mg., and of the other amino acids, 15,500. The addition of other substances to the reaction mixture is indicated in the tables.

The incubation time was 2 hours unless otherwise stated. All glassware and solutions (except plasma) were sterilized in the autoclave. The incorporation of the labeled amino acids was unaffected by penicillin G in concentrations of 10, 100, and 1000 units per ml.

At the end of the incubation the contents of each beaker were added to 80 ml. of water and the reticulocyte protein precipitated with 20 ml. of 35 per cent trichloroacetic acid. If the beaker contained plasma, however, the cells were separated by centrifugation and washed twice with saline to remove plasma proteins, preliminary to the trichloroacetic acid precipitation. On the next day the precipitate was washed by centrifugation ten times with 7 per cent trichloroacetic acid, once with a mixture of equal volumes of acetone and ether, twice with acetone, and finally twice with ether. The washing with acetone and ether removes most of the pigment and gives a product which is easy to grind and spread. The proteins were dried at 55° and counted as previously described (13).

Results

Table I is a summary of the data from consecutive experiments over a number of months on the incorporation of labeled amino acids by reticulocyte proteins of cells incubated either in saline or in plasma. The incorporation in saline is of the same order of magnitude as that observed previously with glycine, leucine, and lysine in rabbit bone marrow cells (15). The amounts of incorporation in plasma were 2 to 3 times greater than in

[3] Heparin sodium obtained from Ben Venue Laboratories, Inc., Bedford, Ohio. Its potency was 110 units per mg.

saline; values of t and P show that the differences are statistically highly significant.

Since plasma contains amino acids, the specific activities of the labeled amino acids added for the incubation were decreased below their original values, and the amounts of incorporation in plasma were, therefore, actually higher than the uncorrected values given in Table I. To correct the plasma values was impracticable, however, since it would have involved amino acid determinations on the plasma used in every experiment. Some idea of the order of magnitude of the error can be derived from Table II, in which amino acid concentrations in the pooled plasma of four anemic

TABLE I

Incorporation of Labeled Amino Acids into Proteins of Rabbit Reticulocytes

The mean values are given.

Labeled amino acid	Glycine*	L-Histidine*	L-Leucine*	L-Leucine†	L-Lysine*
In saline, *μM per gm. protein per hr.*	0.78 ±0.14	1.08 ±0.16	0.70 ±0.16	1.08 ±0.46	0.99 ±0.19
In plasma, *μM per gm. protein per hr.*	1.29 ±0.32	1.75 ±0.29	2.00 ±0.34	3.11 ±1.40	2.51 ±0.82
Plasma less saline, *μM per gm. protein per hr.*	0.50 ±0.25	0.67 ±0.36	1.30 ±0.42	2.03 ±1.06	1.52 ±0.74
Ratio, $\frac{\text{plasma}}{\text{saline}}$	1.64 ±0.28	1.62 ±0.38	2.85 ±0.91	2.87 ±0.80	2.55 ±0.92
No. of comparisons	16	16	26	76	15
t for plasma less saline (14)	8.0	7.5	15.7	16.7	7.9
P	<0.001	<0.001	<0.001	<0.001	<0.001

* Phenylhydrazine only.

† Phenylhydrazine, with folic acid and vitamin B_{12}, used to produce reticulocytosis.

rabbits are given. It should be emphasized that the values in Table II are not necessarily representative or average values; many more analyses will be needed on individual animals to determine the variation. Addition of the labeled amino acids to this particular plasma would approximately halve the specific activity of the glycine, but would leave practically unchanged the specific activities of the histidine, leucine, and lysine.

The injection of folic acid and vitamin B_{12} with phenylhydrazine did not affect the rate of appearance of the reticulocytosis, but the amount of leucine incorporated tended to be somewhat higher in both plasma and saline (Table I). However, the ratio of plasma to saline was the same as when phenylhydrazine only was injected.

Plasma does not lose its accelerating effect after at least several weeks in the deep freeze. When the reticulocytes were hemolyzed by freezing

and thawing, there was no incorporation of leucine either in saline or plasma.

Table III shows the effect of incubation in plasma of different animals or in their non-protein filtrates on incorporation of labeled amino acids into rabbit reticulocyte proteins. The amount incorporated was always greater in plasma than in saline, except in the case of beef plasma, which agglutinated the cells. It is interesting that neither agglutination nor inhibition was observed in calf plasma. The non-protein filtrates referred to in Table III were prepared from plasma by boiling the plasma at pH 5.5, filtering,

TABLE II

Amino Acids in Pooled Plasma of Anemic Rabbits

Amino acid	Concentration	Amino acid	Concentration
	mg. per cent		*mg. per cent*
Alanine	2.0	Lysine	0.8
Arginine	0.5	Methionine	0.5
Aspartic acid	1.1	Phenylalanine	1.3
Cystine	1.3	Proline	1.8
Glutamic acid	3.3	Serine	1.2
Glycine	5.4	Threonine	1.8
Histidine	0.6	Tyrosine	<0.1
Isoleucine	<0.1	Valine	0.8
Leucine	0.4	Ammonia (as NH_3)	0.2

The non-protein filtrate, obtained by boiling the pooled plasma at pH 5.5, was chromatographed on Dowex 50 according to the method of Stein and Moore (16). The amino acids were measured with the ninhydrin reagent against leucine standards, the color was read at 570 mμ, and the chromogenic values of the different amino acids given by Moore and Stein (17) were used in computing the results. The yellow color given by proline with the ninhydrin reagent was measured at 440 mμ against proline standards.

and adjusting the filtrate to pH 8.4. Practically all the accelerating activity of the plasma was present in the non-protein filtrate. The non-protein filtrate of beef plasma was as active as those from plasmas accelerating incorporation.

Plasma which had been ashed and then made up to its original volume with water did not accelerate the incorporation of leucine. Removal of calcium, magnesium, or phosphate, or replacement of all the potassium by sodium in the Krebs-Henseleit incubating solution, had no effect; there was also no effect on addition of glucose or pyruvate to the saline solution or to the saline solution lacking calcium, magnesium, or phosphate. There is, then, no evidence for ascribing the accelerating effect of plasma to its mineral content.

The effect of dilution of plasma on incorporation into the proteins of

washed cell preparations of high, intermediate, and low activities is shown in Table IV. In all three preparations the incorporation was greater at

TABLE III

Effect of Plasma and of Non-Protein Filtrate of Plasma on Incorporation of Labeled Amino Acids into Proteins of Rabbit Reticulocytes

The results are expressed as per cent of value in saline.

Labeled amino acid	Incubation solution	Per cent of saline value
L-Leucine	Saline	100
	Anemic adult rabbit plasma	259
	" " " non-protein filtrate	212
	Calf plasma	222
	" non-protein filtrate	199
	Beef plasma	45
	" non-protein filtrate	205
	Horse plasma	216
	" non-protein filtrate	172
	Normal adult rabbit plasma	300
	" young " "	249
	Rat plasma	225
	Sheep plasma	178
	Human "	255
L-Histidine	Anemic adult rabbit plasma	161
	" " " non-protein filtrate	145
L-Lysine	" " plasma	230
	" " " non-protein filtrate	205

TABLE IV

Effect of Concentration of Plasma on Incorporation of Labeled Leucine into Rabbit Reticulocyte Proteins

Volume of plasma in 4.0 ml. of reaction mixture, V_s	Fraction of plasma present, $\frac{V_s}{4.0}$	Rates in μM per gm. protein per hr.			$\frac{R_1}{R_2}$	$\frac{R_2}{R_3}$
		Experiment 1, R_1	Experiment 2, R_2	Experiment 3, R_3		
ml.						
0	0	1.97	1.19	0.66	1.65	1.80
0.3	0.075		1.76	0.88		2.00
0.8	0.20	5.08	2.60	1.28	1.95	2.03
1.7	0.42	6.64	3.54	1.51	1.87	2.34
2.5	0.62	8.84	4.05	1.81	2.18	2.25
3.3	0.82	10.01	4.53	1.90	2.20	2.38

higher plasma concentrations. The relative activities of the preparations persisted at all dilutions.

Results of experiments in which the volume of cells used was increased

above the usual 0.5 ml. are given in Table V. Although the total amount of leucine incorporated was greater with the increased number of cells, the amount of incorporation per gm. of protein was essentially constant. Other experiments were carried out to test the effect of washing on the cells. The incorporation was the same with whole blood as with corresponding

TABLE V

Effect of Concentration of Reticulocytes on Incorporation of Labeled Leucine

Cell suspension	Saline	μM per gm. protein per hr.	
		Experiment 1	Experiment 2
ml.	*ml.*		
2.8	1.2	1.23	1.07
1.9	2.1	1.00	1.20
1.2	2.8	1.25	1.22
0.7	3.3	1.39	1.16

TABLE VI

Falling Off with Time of Incorporation of Labeled Leucine in Plasma and in Saline

The results are expressed as micromoles per gm. of protein.

Time	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	Saline	200 mg. per cent glucose in saline	Saline	0.01 M succinate in saline	Plasma	Plasma + 100 mg. per cent glucose	Plasma	0.01 M succinate in plasma
hrs.								
0	0	0	0	0	0.13	0.12	0.14	0.14
1	1.56	1.56	0.70	0.73	3.82	4.44	3.53	3.54
2	2.28	2.49	1.12	1.12	6.48	6.08	4.13	4.36
3	3.19	3.28	1.43	1.50	6.83	7.04	5.11	4.58
4	3.76	3.74	1.79	1.91	7.91	7.89	4.71	5.31

amounts of washed cells and plasma. Cells washed one, two, three, or four times gave the same incorporation, both in saline and in plasma.

The rate of incorporation of leucine, both in saline and in plasma, decreased progressively during a 4 hour incubation (Table VI). Addition of glucose or succinate did not affect either the absolute rate or the rate of decrease.

The amount of incorporation of leucine in a red blood cell preparation increased with increased reticulocytosis (Table VII). In a more detailed study Holloway and Ripley[4] found that the degree of incorporation and

[4] In this laboratory; see the accompanying paper.

the concentrations of ribose nucleic and desoxyribose nucleic acids in the cells were all proportional to the extent of reticulocytosis.

A summary of the effects of various inhibitors on the incorporation of glycine, histidine, leucine, and lysine, both in plasma and in saline, is given in Table VIII. The effect of anaerobiosis on the incorporation of any one amino acid was extremely variable. With some preparations there was no inhibition; with others, a marked inhibition. Other inhibitors gave more consistent results. The values in Table VIII for each inhibitor are averages of six to ten experiments. There were no striking differences among

TABLE VII

Effect of Degree of Reticulocytosis on Incorporation of Labeled Leucine into Proteins of Rabbit Reticulocytes

The results are expressed as micromoles per gm. of protein per hour; reticulocytes in per cent red blood cells.

Animal No.		No. of days of phenylhydrazine injection				
		0	3	6	7	10
1	Reticulocytes	0.1	4.7		67	90
	Leucine incorporated in plasma	0.07	0.62		4.10	3.80
	" " " saline	0.07	0.36		1.87	2.49
2	Reticulocytes	0.1	9.3		77	90
	Leucine incorporated in plasma	0.02	2.35		4.77	5.19
	" " " saline	0.07	1.05		2.63	2.77
3	Reticulocytes	0.1	20	81		
	Leucine incorporated in plasma	0.05	2.01	7.75		
	" " " saline	0.03	0.86	2.89		
4	Reticulocytes	0.1	22	82		
	Leucine incorporated in plasma	0.04	2.34	6.23		
	" " " saline	0.02	0.91	2.60		

the amino acids in the effects of the inhibitors. From the inhibitor picture as a whole, it appears, then, that the process of incorporation is the same for all four amino acids.

Reticulocytes appear to differ from marrow cells in that, with the latter, anaerobiosis completely inhibits the incorporation of glycine, leucine, and lysine (15). Respiration appears to be only indirectly associated with the process of amino acid incorporation, since anaerobiosis, diethyl dithiocarbamate, and α,α'-dipyridyl are only somewhat inhibitory. In this respect rabbit reticulocytes differ from other preparations previously studied (1, 18–20). The process in reticulocytes resembles the incorporation of lysine into guinea pig liver homogenate (13) and of other amino acids into glutathione (21).

In order to determine that the labeled amino acid was not merely ad-

sorbed onto the protein and also to obtain information on the mode of incorporation, the proteins obtained after incubation with the labeled amino acids were subjected to various treatments (Table IX). The proteins obtained from glycine, histidine, and lysine incubations were the total reticulocyte proteins, chiefly globin. The leucine-labeled protein was hemoglobin obtained from the reticulocytes by the dialysis method of Drabkin (22). Heating the proteins for 15 minutes at 90° in 5 per cent trichloroacetic acid, followed by four washings with 5 per cent trichloroacetic acid, did not

TABLE VIII

Effect of Inhibitors on Incorporation of Labeled Amino Acids into Proteins of Rabbit Reticulocytes

The results are expressed as per cent of that without inhibitor.

Inhibitor	Concentration	Glycine		Histidine		Leucine		Lysine	
		Plasma	Saline	Plasma	Saline	Plasma	Saline	Plasma	Saline
	M								
Anaerobiosis		45	67	77	24	37	43	75	100
Arsenate	10^{-3}	71	56	82	54	80	87	54	86
Arsenite	10^{-3}	10	9	5	7	4	12	3	9
Azide	10^{-3}	70	63	81	85	87	95	70	91
Diethyl dithiocarbamate	10^{-3}	81	59	89	100	63	92	47	100
α,α'-Dipyridyl	10^{-3}	36	50	54	58	38	75	31	69
2,4-Dinitrophenol	10^{-3}	7	11	17	10	19	12	14	21
Fluoride	2×10^{-2}	1	0	3	6	1	9	0	0
"	2×10^{-3}					99	94		
Hydroxylamine	2×10^{-2}	11	16	11	40	7	11	5	32
"	2×10^{-3}	100	100	100	100	99	100		
Iodoacetate	2×10^{-3}	12	40	46	76	49	51	21	76
Ammonium molybdate	2×10^{-3}	42	16	55	100	61	74	33	70
" "	2×10^{-4}					98	90		

remove any radioactivity. The counts were also not decreased by dialysis, in the presence of a weight of the corresponding unlabeled L-amino acid equal to that of the protein, against 0.1 M sodium carbonate. That the labeled proteins did not contain free labeled amino acids was also shown by the fact that no radioactive carbon dioxide was liberated on treatment with ninhydrin (23, 24). Oxidation of the proteins with performic acid according to the method of Toennies and Homiller (25) did not decrease the count. Results of treatment of the glycine-, leucine-, and lysine-labeled proteins with dinitrofluorobenzene according to the method of Sanger (26) indicated that the labeled amino acids in the proteins did not have free amino groups.

The following procedures were used for the identification and isolation of the radioactive amino acids from the proteins.

Glycine and Serine—2.000 gm. of protein from the incubations with labeled glycine, 1.000 gm. of inactive carrier glycine, and 1.000 gm. of inactive carrier DL-serine were refluxed for 21 hours with 6 N HCl and then chromatographed on Dowex 50 (16). The glycine fraction was converted to acetylglycine (27). The specific activity of the acetylglycine on successive recrystallizations was 4.42, 4.30, 4.00, 3.90, and 3.98 c.p.m. per mg. Only 10 per cent remained after the last recrystallization; m.p. 207–208°.

Calculated, C 41.02, H 6.03, N 11.96; found, C 40.83, H 5.94, N 11.85

TABLE IX

Results of Treatment of Proteins Obtained after Incubation with C^{14}-Carboxyl-Labeled Glycine, L-Histidine, L-Leucine, or L-Lysine

Treatment	Labeled amino acid used in incubation			
	Glycine*	Histidine*	Leucine†	Lysine*
None; radioactivity of protein, c.p.m. per mg.	4.30	7.10	14.3	6.87
Heated with 5% trichloroacetic acid; specific activity of protein as % of that of original protein	101	95	99	95
Dialyzed; specific activity of protein as % of that of original protein	101	98	99	100
Treated with ninhydrin; counts in liberated CO_2 as % of that originally in protein	0	1	1	0
Treated with performic acid; specific activity of protein as % of that of original protein	108	106	109	101
Hydrolysis; % of count originally in protein found in isolated amino acid corresponding to that with which it had been incubated	Glycine 79; serine 14	101	103	99
% radioactivity found in labeled amino acid which has its amino group free in protein	1		1	1

* Total proteins of the reticulocytes used.

† Hemoglobin only used.

The bulk of the HCl was removed from the serine fraction, which contained also aspartic acid, threonine, and glutamic acid, by several evaporations *in vacuo*; the residue was dissolved in water, brought to pH 5.8 with ammonium hydroxide, and electrodialyzed, as previously described, for 7 hours. The combined anolyte fractions, consisting of crude aspartic and glutamic acids, contained a total of only 100 c.p.m. The serine was isolated from the catholyte and from the solution in the center compartment as the dinitrophenyl derivative (28). The specific activity of the dinitrophenyl derivative after successive recrystallizations from methanol was 0.38, 0.48, and 0.39 c.p.m. per mg. Only 10 per cent remained after the last recrystallization; m.p. 194–196° (uncorrected).

Calculated, C 39.86, H 3.34, N 15.49; found, C 39.89, H 3.47, N 15.31

The original protein contained a total of 8600 c.p.m. If it is assumed that the protein was hemoglobin and that rabbit hemoglobin has the same composition as human hemoglobin (29), then the protein contained 100 mg. each of glycine and serine. 79 per cent of the count of the original protein was, therefore, accounted for as glycine; *i.e.*, 1100 × 1.56 (factor for converting glycine to acetylglycine) × 3.96 = 6795 c.p.m. 14 per cent of the count of the original protein was accounted for as serine; *i.e.*, 1100 × 2.58 (factor for converting serine to 2,4-dinitrophenol serine) × 0.415 = 1179 c.p.m.

Histidine—1.000 gm. of protein from the incubations with labeled histidine and 0.740 gm. of inactive carrier L-histidine were hydrolyzed. The hydrolysate was evaporated to dryness and then electrodialyzed as previously described. The combined catholytes were brought to pH 5.6 and again electrodialyzed. Histidine was precipitated at pH 7.4 as the silver salt, after removal of ammonia by a stream of nitrogen from the combined catholytes. The silver salt was decomposed with hydrogen sulfide in the presence of sulfuric acid, the sulfate was removed by adding barium hydroxide to pH 4.0, and the histidine was precipitated from 50 per cent methanol as the nitranilate. The specific activity of the nitranilate after successive recrystallizations was 3.64, 3.53, and 3.58 c.p.m. per mg.

Calculated, C 37.50, N 18.22; found, C 37.45, N 18.20

The original protein contained a total of 7100 c.p.m. If the assumption is made that the protein was hemoglobin and of the same composition as human hemoglobin (29), then the protein contained 80 mg. of histidine. 101 per cent of the count of the original protein was, therefore, accounted for as histidine, *i.e.*, 820 × 2.476 (factor for converting histidine to the nitranilate) × 3.55 = 7208 c.p.m.

Leucine—299 mg. of hemoglobin from the incubations with labeled leucine

and 299 mg. of inactive carrier L-leucine were hydrolyzed. After removal of excess acid, the hydrolysate was chromatographed on starch (17, 30); the leucine-isoleucine-phenylalanine fraction from the starch column was rechromatographed on Dowex 50 (16). Crystalline leucine was obtained by lyophilization of the leucine fraction. It was recrystallized by dissolving in hydrochloric acid and precipitating with ammonia at pH 5.0. The specific activity of the leucine after successive recrystallizations was 12.8, 12.9, and 12.9 c.p.m. per mg.

The original hemoglobin contained a total of 4279 c.p.m. If the assumption is made that the hemoglobin is of the same composition as human hemoglobin (29), then it contained 45 mg. of leucine. 103 per cent of the count of the original hemoglobin was, therefore, accounted for as leucine; *i.e.*, 344 × 12.87 = 4428 c.p.m.

Lysine—1.000 gm. of protein from the incubations with labeled lysine and 0.800 gm. of L-lysine were hydrolyzed with hydrochloric acid. After removal of excess acid, the hydrolysate was electrodialyzed, as previously described, for 7 hours. The combined catholytes were acidified to pH 4.4 and again electrodialyzed for 3 hours. After removal of ammonia by a stream of nitrogen the combined catholytes were adjusted to pH 6.8 with a saturated ethanol solution of picric acid. The specific activity of the lysine picrate, after successive recrystallizations from water, was 3.01, 3.11, and 3.00 c.p.m. per mg.

Calculated, N 18.66; found, N 18.86

The original protein contained a total of 6870 c.p.m. On the assumption that the protein was hemoglobin and of the same composition as human hemoglobin (29), the protein contained 80 mg. of lysine. 99 per cent of the count of the original protein was, therefore, accounted for as lysine; *i.e.*, 880 × 2.567 (factor for converting lysine to lysine picrate) × 3.00 = 6777 c.p.m.

All the data of Table VIII are in accord with the interpretation that rabbit reticulocytes, when incubated with labeled amino acids, incorporate them into their proteins. In the case of glycine, it is evident that the reticulocytes convert part of the glycine to serine, which is then also incorporated.

Effect of Amino Acids and of Other Substances on Incorporation—Since the accelerating effect of plasma on incorporation of labeled amino acids was not due to its protein or to its mineral content, it seemed possible that the effect might be due to the amino acids present. A systematic study of the effects of additions of various amino acids was, therefore, begun, after it was shown that an acid hydrolysate of casein increased incorporation in saline.

The mixture of amino acids referred to as total amino acids gave the following concentrations (mg. per cent) in the incubating medium (in all experiments unlabeled amino acid corresponding to the labeled amino acid used in the incubation was omitted): L-alanine, 2.06; L-arginine, 1.65; L-aspartic acid, 2.29; L-cystine, 0.13; L-glutamic acid, 8.74; glycine, 0.19; L-histidine, 1.09; L-hydroxyproline, 0.56; DL-isoleucine, 4.73; L-leucine, 3.64; L-lysine, 3.06; L-methionine, 1.28; DL-norleucine, 4.73; L-phenylalanine, 1.88; L-proline, 2.89; L-serine,[5] 2.89; L-threonine, 1.43; L-tryptophan, 0.45; L-tyrosine, 2.51; L-valine, 2.44.[6] Whenever, in later experiments cited, an unlabeled amino acid was added to the incubating medium separately or in other combination than the above mixture, its concentration was the same as in the total amino acid mixture.

The amounts of incorporation of each of the four labeled amino acids was greater in plasma or in saline containing total amino acids than in saline

TABLE X

Effect of Other Amino Acids on Incorporation of Labeled Amino Acids into Rabbit Reticulocytes

Micromoles per gm. of protein per 2 hours.

	Labeled amino acid in reaction mixture			
	Glycine	Histidine	Leucine	Lysine
Saline alone	1.59	2.49	1.46	1.96
Plasma "	2.56	3.09	3.26	4.91
Saline + total amino acids	2.80	3.41	2.48	3.71

(Table X). The values given are uncorrected for the dilution of the labeled amino acids by the unlabeled amino acids already present in the plasma; in the case of glycine, therefore, which is present in relatively large amounts in the plasma (Table II), the true incorporation is probably greater in the plasma than in the saline containing total amino acids. The incorporation of leucine and of lysine was also greater in plasma than in saline containing total amino acids. This finding suggested that plasma contains an accelerating factor other than amino acids, since the concentrations of most of the amino acids were higher in the total amino acid mixture than in plasma.

[5] Prepared and kindly given to us by Dr. C. E. Harrold.

[6] L-Alanine and L-threonine were obtained from the Bios Laboratories, Inc., L-arginine, L-glutamic acid, and L-methionine from the H. M. Chemical Company, Ltd., L-aspartic acid from the Pfanstiehl Chemical Company, L-cystine, L-hydroxyproline, and DL-norleucine from the Amino Acid Manufactures, University of California at Los Angeles, glycine, L-histidine, DL-isoleucine, L-leucine, L-lysine, L-proline, and L-tyrosine from Merck and Company, Inc., L-phenylalanine and L-valine from the Nutritional Biochemicals Corporation, L-tryptophan from the Van Camp Laboratories.

The effect of omitting each amino acid individually from the total amino acid mixture is shown in Table XI. The omission of histidine, phenylalanine, or valine from the mixture containing all the remaining amino acids decreased the accelerating effect of the total amino acid mixture. The omission of tryptophan and tyrosine gave a slight and variable reduction which was within the experimental error. The total amino acid mixture from which all the other amino acids not listed in Table XI were individually omitted gave the same accelerating effect as the total amino acid mixture.

TABLE XI

Effect of Other Amino Acids on Incorporation in Saline

The results are expressed as per cent of value with total amino acid mixture.

Unlabeled amino acids added	Labeled amino acids incorporated			
	Glycine	Histidine	Leucine	Lysine
None	67	77	58	51
Total amino acids	100	100	100	100
" " " less histidine	46		64	62
" " " " leucine	89	96		91
" " " " phenylalanine	75	66	78	86
" " " " tryptophan	95	92	98	88
" " " " tyrosine	95	97	90	85
" " " " valine	75	71	61	63
" " " " each amino acid separately except those cited	92–107	94–102	95–103	97–103

TABLE XII

Effect of Individual Amino Acids on Incorporation of Labeled Leucine

The results are expressed as per cent of value in saline alone.

Saline	100
" + histidine	160
" + total amino acids	198
Plasma (no amino acids added)	307
Saline + each amino acid separately except histidine	87–110

The effect of adding each amino acid individually to the saline solution was tested (Table XII). Only histidine accelerated the incorporation of leucine; none of the other amino acids of the total amino acid mixture had any effect. This result supports the interpretation that phenylalanine and valine will accelerate the incorporation only in the presence of histidine.

To determine whether any other amino acid was synergistic in the incorporation of leucine and also to find an optimal amino acid mixture, a mixture of histidine, phenylalanine, tyrosine, and valine was tested with each

of the other amino acids of the total amino acid mixture in turn (Table XIII). The mixture of histidine, phenylalanine, tyrosine, and valine was as effective as the total amino acid mixture, and addition of the other amino acids individually, or of the remaining indispensable amino acids, or of all the dispensable amino acids was without significant effect. Increasing the concentrations of histidine, phenylalanine, and valine in the saline to 2 or 4 times the usual concentrations, either separately or together, did not further increase leucine incorporation. The higher concentrations were somewhat inhibitory.

Experiments on the incorporation of labeled histidine in saline gave re-

TABLE XIII

Effect of Histidine, Phenylalanine, Tyrosine, and Valine on Incorporation of Labeled Leucine in Saline

The results are expressed as per cent of value in saline alone.

Saline	100
Plasma (no amino acids added)	318
Saline + total amino acids	170
" + " " " less (histidine + phenylalanine + tyrosine + valine)	80
Saline + histidine + phenylalanine + tyrosine + valine	169
Saline + histidine + phenylalanine + tyrosine + valine + each of remaining amino acids separately	155–180
Saline + histidine + phenylalanine + tyrosine + valine + isoleucine + methionine + norleucine + arginine + threonine + tryptophan	165
Saline + histidine + phenylalanine + tyrosine + valine + alanine + aspartic acid + cystine + glutamic acid + glycine + hydroxyproline + proline + serine	160

sults similar to those obtained with labeled leucine (Tables XII and XIII). Addition of the total amino acid mixture to saline gave a 25 to 40 per cent acceleration, each amino acid had no effect, a mixture of phenylalanine, tyrosine, and valine was as effective as the total amino acid mixture, and the effectiveness of the mixture was not augmented by the addition of any other single amino acid.

When plasma was used as the incubating medium, the effect of adding amino acids on the incorporation of histidine differed from that obtained in saline (Table XIV). Leucine, phenylalanine, and valine separately increased the incorporation somewhat; other individual amino acids did not. The stimulation by a mixture of phenylalanine, tryptophan, tyrosine, and valine, which was practically the sum of the individual effects, was far less than that of the total amino acid mixture. The addition of leucine to the

mixture of the four amino acids gave an acceleration equal to that of the total amino acid mixture; the sum of the stimulation by leucine, phenylalanine, tryptophan, tyrosine, and valine individually was less than their combined effect. Tryptophan and tyrosine probably do not participate in the synergistic action.

The effect of the omission from the total amino acid mixture of each amino acid individually was also tested with labeled histidine in plasma (Table XIV). Omission of leucine alone from the total amino acid mixture gave a rate little more than that in plasma; omission of either phenylalanine or valine also reduced the incorporation to some extent. When each of the

TABLE XIV

Effect of Other Amino Acids on Incorporation of Labeled Histidine in Plasma

The results are expressed as per cent of value in saline alone.

Saline	100	Saline	100
Plasma alone	165	Plasma alone	178
" + leucine	203	" + total amino acids	373
" + phenylalanine	199	" + " " " less leucine	186
" + tryptophan	173	Plasma + total amino acids less phenylalanine	263
" + tyrosine	172	Plasma + total amino acids less tryptophan	360
" + valine	200	Plasma + total amino acids less tyrosine	360
" + each amino acid separately except those cited above	160–168	Plasma + total amino acids less valine	303
Plasma + total amino acids	460	Plasma + total amino acids less each amino acid separately but always including those cited above	364–381
" + phenylalanine + tryptophan + tyrosine + valine	248		
Plasma + leucine + phenylalanine + tryptophan + tyrosine + valine	419		

other amino acids of the total amino acid mixture was individually omitted, the incorporation was the same as in the complete mixture. This synergistic effect of leucine was not observed in saline; it evidently depends on the presence in plasma of another factor.

Experiments on the effect of added amino acids on the incorporation of labeled glycine, histidine, leucine, and lysine, both in plasma and in saline, are summarized in Table XV. A mixture of histidine, phenylalanine, tryptophan, and valine was as effective as the total amino acid mixture in either saline or plasma (except in histidine incorporation in plasma). When leucine was added to the mixture of the five amino acids, the incorporation of glycine and lysine in plasma was more than doubled; in saline, however, leucine had no synergistic effect. Leucine alone had no accelerating effect in saline and only a relatively small one in plasma.

We have found, in experiments with guinea pig liver mitochondria (31), rabbit bone marrow cells (15), and rat diaphragm (1), that the sum of the counts of the protein obtained by incubation with each of labeled glycine, leucine, or lysine separately is equal to the count of the protein obtained by incubation with a mixture of the three amino acids. A similar additive effect is found with rabbit reticulocytes, in both saline and in plasma, with labeled glycine, leucine, and lysine (Table XVI). However, in all combinations containing labeled histidine, the count of the protein was a little

TABLE XV

Effect of Other Amino Acids on Incorporation of Labeled Glycine, Histidine, Leucine, and Lysine

Micromoles per gm. of protein in 2 hours.

Unlabeled addition	Labeled amino acid							
	Glycine		Histidine		Leucine		Lysine	
	Plasma	Saline	Plasma	Saline	Plasma	Saline	Plasma	Saline
None	2.56	1.59	3.09	2.49	3.23	1.45	4.91	1.96
Leucine	2.74	1.61	3.82	2.39			6.10	1.81
Histidine + phenylalanine + tryptophan + tyrosine + valine	2.97	3.07	3.76	3.25	6.76	2.68	5.38	3.44
Total amino acids	2.70	2.80	8.64	3.49	6.26	2.70	5.48	3.71
Leucine + histidine + phenylalanine + tryptophan + tyrosine + valine	6.32	2.90	7.86	3.00			12.83	3.91

The unlabeled amino acid corresponding to the labeled amino acid used was always omitted from the added unlabeled amino acids.

greater than the expected sum; this acceleration by histidine is in accord with the data of Tables XI, XII, XIII, and XV.

A number of substances other than amino acids were tested for an accelerating effect on the incorporation of leucine in saline. The following had no effect: acetate (0.01 M), adenine (0.75 mg. per cent), adenosine (1.5 mg. per cent), adenylic acid (25 mg. per cent), bovine serum albumin (7 per cent), ascorbic acid (33 and 3.3 mg. per cent), adenosinetriphosphate (ATP) (0.001 M), coenzyme A (25 units per ml.), coenzyme II (0.001 M), creatine (0.75 mg. per cent), folic acid (10 γ per ml.), folinic acid (10 and 1 γ per ml.), glucose (0.01 M), glutathione (300 mg. per cent), glycocyamine (0.75 mg. per cent), guanine (0.75 mg. per cent), α-ketoglutarate (0.01 M), peptone, Difco Nos. 1, 2, 3, and 4 (100 mg. per cent), peptone, Witte (100 mg. per cent), pyruvate (0.01 M), riboflavin (3.0 and 0.3 mg. per cent), succinate (0.01 M), thiamine (0.3 and 0.03 mg. per cent). The following were

inhibitory: citrate (0.01 M), vitamin B_{12} (1 and 0.1 γ per ml.), and malate (0.01 M).

Some of the above compounds were tested in saline containing the total amino acid mixture. The following were without effect: adenylic acid (25 mg. per cent), a mixture of ATP (0.001 M) and glucose (0.01 M), citrulline (20 mg. per cent), creatine (1 mg. per cent), glucose (0.01 M), glutamine (3.9 mg. per cent), malate (0.01 M), and pyruvate (0.01 M). The following

TABLE XVI

Incorporation of Labeled Glycine, L-Histidine, L-Leucine, and L-Lysine When Incubated Separately and Together

Labeled amino acids	Incubated in saline				Incubated in plasma			
	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	Observed	Observed / Calculated	Observed	Observed / Calculated	Observed	Observed / Calculated	Observed	Observed / Calculated
	c.p.m. per mg. protein	*per cent*	*c.p.m. per mg. protein*	*per cent*	*c.p.m. per mg. protein*	*per cent*	*c.p.m. per mg. protein*	*per cent*
Glycine	4.4		4.2		7.65		10.0	
Histidine	8.0		7.0		11.9		15.1	
Leucine	4.65		4.3		9.2		10.5	
Lysine	4.2		3.7		9.2		10.6	
Glycine + histidine + leucine	21.6	127	20.4	132	31.3	109	41.5	131
Glycine + leucine + lysine	13.35	101	11.8	97	25.4	98	31.4	101
Glycine + histidine + lysine	20.3	122	19.3	130	30.4	106	38.5	108
Histidine + leucine + lysine	23.4	139	19.7	131	30.4	100	39.1	108
Glycine + histidine + leucine + lysine	28.2	133	25.8	134	40.1	106	53.0	115

were inhibitory: citrate (0.01 M), α-ketoglutarate (0.01 M), succinate (0.01 M), and a mixture of malate (0.01 M) and pyruvate (0.01 M). A slight acceleration (10 to 15 per cent) was observed in saline with coenzyme I (CoI) (0.001 M) and with ATP (0.001 M). A mixture of CoI and ATP was no more effective than either substance alone.[7]

Table XVII presents more evidence of an accelerating factor in addition

[7] No conclusions are to be drawn from the effects of substances other than the amino acids noted above. They are only observations on the test system as it was used in these experiments. We have indications that under other conditions different results may be obtained in some cases.

to amino acids in the non-protein filtrate of plasma. The data show that the acceleration by the mixture of histidine, leucine, phenylalanine, tryptophan, tyrosine, and valine, was greater in the non-protein filtrate of plasma than in saline.

An accelerating factor is present also in the non-protein filtrates from

TABLE XVII

Effect of Other Amino Acids Added to Non-Protein Filtrate of Anemic Rabbit Plasma on Incorporation of Labeled L-*Leucine and* L-*Lysine*

The results are expressed as per cent of value in saline alone.

Addition	Labeled amino acid			
	Leucine		Lysine	
	Saline	Non-protein filtrate	Saline	Non-protein filtrate
None	100	207	100	246
Histidine, leucine,* phenylalanine, tryptophan, tyrosine	162	331	199	462

* Unlabeled leucine was added only in the experiments with labeled lysine.

TABLE XVIII

Accelerating Effect of Non-Protein Filtrate of Red Cells on Incorporation of Labeled L-*Lysine into Proteins of Rabbit Reticulocytes*

The results are expressed as per cent of value in saline.

Red cell filtrate of	Per cent of value in saline
Beef	158
Guinea pig	166
Hog	150
Horse	153
Human	213
Rabbit	185
" reticulocytes	207
Sheep	152

the normal erythrocytes of beef, guinea pig, hog, horse, man, rabbit, and sheep, and from rabbit reticulocytes (Table XVIII). The cells, separated from the plasma of the heparinized blood by centrifugation, were washed twice with saline and then frozen. The mixture containing the frozen cells and an equal volume of saline was brought to pH 5.5 and boiled; the non-protein filtrate obtained by filtration was brought to pH 8.4. The mixture of unlabeled histidine, leucine, phenylalanine, tryptophan, tyrosine, and valine was added to both the saline and red cell filtrate reaction mixtures.

Since the data indicate the presence of an accelerating factor in normal erythrocytes, their failure to incorporate labeled amino acids may be due to lack of either the enzyme system or of necessary organization of the cell proteins.

The presence of inhibitory as well as of accelerating factors in non-protein filtrates of plasma was detected by the chromatopile method of Mitchell and Haskins (32). A mixture of *n*-propanol and N acetic acid (3:1) was the developing solvent. The residues obtained after division of the pile into nine equal fractions, elution of each with water, and concentration of the aqueous extracts to dryness, were tested for their accelerating effect

TABLE XIX

Effect of Extracts of Animal Tissues of Yeast and of Escherichia coli on Incorporation of Labeled Lysine

Source of tissue extract	Per cent of value in saline		
	Extract in 4.0 ml. of reaction mixture		
	2.7 ml.	0.5 ml.	0.3 ml.
Duodenum, dried at 40°, hog	1	34	61
Kidney, " " 40°, "	5	84	94
Stomach lining, dried at 40°, hog	5	50	76
Blood, whole, horse	242	142	135
Liver, dried at 75°, hog	100	214	250
Spleen, " " 37°, "	6	134	166
Yeast, bakers'	67		160
" brewers'	41		113

All the dried tissues were obtained from the VioBin Corporation, Monticello, Illinois.

on incorporation of labeled leucine and lysine. The mixture of unlabeled histidine, leucine, phenylalanine, tryptophan, tyrosine, and valine was added to both the saline and the test reaction mixtures. The top fractions accelerated the incorporation; the lower fractions inhibited the incorporation, the third fraction from the bottom being the most inhibitory. The inhibition cannot be accounted for by the decrease in specific activity of the leucine or of the lysine (Table II).

Evidence of both inhibitory and accelerating activity in extracts of various animal tissues and of yeast is given in Table XIX. Non-protein filtrates from the dried animal tissues were obtained by filtering a boiled suspension of 20 gm. of tissue and 100 ml. of H_2O, previously adjusted to pH 5.5; the pH of the filtrate was adjusted to 8.4 for testing. The horse blood extract was prepared by adding an equal volume of saline to the frozen whole blood; the mixture was boiled at pH 5.5 and filtered, and the

pH of the filtrate adjusted to 8.4. The brewers' and bakers' yeasts, after two preliminary washings, were treated by the same procedure as the blood. The results in Table XIX indicate a high concentration of inhibitory factor (or factors) in duodenum, kidney, and stomach lining and the presence of both inhibitory and accelerating factors in liver, spleen, and yeast, with a greater reduction on dilution of the inhibitory effect than of the accelerating effect. Blood contains predominantly the accelerating factor, and dilution, therefore, gives only a reduction of acceleration. As with the non-protein plasma filtrate, the inhibitory effects of the tissue extracts cannot be ascribed to reduction in specific activity of labeled lysine by unlabeled lysine in the extracts.

The properties of the accelerating factor in anemic rabbit plasma are being investigated. Boiling at pH 5.5 for 15 minutes, heating for several

TABLE XX

Effect of Preincubation of Reticulocytes on Incorporation of Labeled Lysine

The results are expressed as micromoles per gm. of protein.

Preincubation time	Incorporation during subsequent 2 hrs. in		
	Saline alone	Saline with added liver extract	Increase due to liver extract
hrs.			
0	2.22	6.14	3.92
2	0.91	4.20	3.29
3	0.56	3.06	2.50
4	0.41	2.22	1.81

hours at 70°, lyophilization, or storage at deep freeze temperatures does not decrease the activity. The activity is lost by refluxing for 20 hours with 6 N hydrochloric acid, but not by standing overnight at room temperature in normal hydrochloric acid. Extraction of the non-protein filtrate with ether or chloroform did not remove activity. The ultrafiltrate of plasma had only slight activity; the dialysate of the non-protein filtrate at pH 5.5 was very active. The factor could be adsorbed on Lloyd's reagent at pH 2.0 and eluted at pH 10.0. On electrodialysis of the accelerating factor in liver nearly all the activity remains in the neutral compartment. On digestion of the liver factor for 12 hours at 38° with 0.1 per cent carboxypeptidase, chymotrypsin, pepsin, or trypsin very little activity was lost.

It was shown in Table VI that the rate of incorporation of leucine became progressively slower during incubation. This phenomenon was investigated further in experiments such as the following. The washed cells were preincubated at 38° under 95 per cent O_2 and 5 per cent CO_2 in Krebs-

Henseleit solution containing histidine, leucine, phenylalanine, tryptophan, tyrosine, and valine. After time intervals indicated in Table XX labeled lysine, with or without 0.5 ml. of liver extract, prepared as above, was added and the incubation continued for 2 hours more. Table XX shows that there was a progressive loss of ability of the cells to incorporate lysine, and that a large part, but not all, of this ability was restored by the liver extract. These results indicate that one of the necessary factors lost in the cells in the preincubation is the non-amino acid-stimulating factor, and that the activity of the cells in saline alone depends on their content of this factor. The non-protein filtrate or liver extract accelerated incorporation in cells, whether used directly or preincubated, because each supplied, in the amounts used, approximately 5 times the amount of the stimulating factor initially present in the cells, thus replenishing, if not actually increasing, the concentration of the factor lost in the cells during incubation. None of the factor is lost during incubation without cells.

DISCUSSION

The literature on the relation between amino acids and blood formation in animals is extensive (33–55), but the results reported are unsystematic and some are conflicting. Nevertheless, it appears that there may be a relationship between labeled amino acid incorporation into reticulocyte proteins and hemoglobin synthesis.

For example Sebrell (53) found that omission of some of the essential amino acids from the diet of rats made anemic by bleeding handicapped red cell regeneration more than the omission of others. The amino acids found to be most necessary for red cell and hemoglobin regeneration, stated in order of their effectiveness, were histidine, valine, lysine, phenylalanine, and leucine; the list agrees surprisingly well with the amino acids accelerating incorporation of labeled amino acids into rabbit reticulocyte proteins.

Nizet and Robscheit-Robbins (54) found that dog reticulocytes did not mature so quickly *in vitro* in the blood of dogs with hemorrhagic anemia and hypoproteinemia as in normal blood, unless a mixture of the ten essential amino acids and glycine was added to the anemic blood. Variations from animal to animal were too great for any conclusions to be drawn regarding differences among the individual amino acids. Orten and Orten (48) studied the effects on hemoglobin regeneration of adding individual amino acids to the diet of young rats previously made anemic by a low protein diet. They concluded that there was no "key" amino acid; however, some amino acids appeared to be more effective than others in their experiments.

One general conclusion that can be drawn is that, although all the amino acids in hemoglobin are, of course, needed for its synthesis, some amino

acids accelerate hemoglobin synthesis more than others. A similar conclusion can be drawn regarding the influence of certain amino acids on the incorporation of other labeled amino acids into rabbit reticulocyte proteins.

SUMMARY

1. The synthesis of L-histidine labeled with C^{14} in the carboxyl group is described.

2. The incorporation *in vitro* of labeled glycine, L-histidine, L-leucine, and L-lysine into rabbit reticulocytes, both individually and as a mixture, was studied. Evidence is given for the presence of labeled serine in the protein of reticulocytes incubated with labeled glycine, for the absence in the labeled proteins of adsorbed labeled amino acids, and for the absence in the labeled proteins of labeled amino acids with free amino groups. The effect on the incorporation of some oxidation and of some phosphorylation inhibitors was studied.

3. The incorporation is greater when the reticulocytes are incubated in plasma than when they are incubated in saline. Histidine, phenylalanine, and valine, in low concentrations, accelerate the incorporation both in saline and in plasma; the accelerating effect of the plasma is not, however, due solely to its content of these amino acids. In plasma, but not in saline, leucine exerts a large synergistic action on the accelerating effect of the other amino acids. The evidence, therefore, points to the presence in plasma of a factor accelerating the incorporation of labeled amino acids.

4. The accelerating factor is in the non-protein fraction of plasma. Accelerating factors were found in the plasma of every mammal investigated, in their normal erythrocytes, in rabbit reticulocytes, liver, spleen, and yeast.

5. Some properties of the accelerating factors are given. They are not known amino acids or cofactors.

6. When reticulocytes are incubated in saline with the accelerating amino acids for 2 to 3 hours at 38°, they lose most of their ability to incorporate labeled amino acids. A large part of this lost activity is restored by the addition (after preincubation) of an aqueous extract of liver.

BIBLIOGRAPHY

1. Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H., *J. Biol. Chem.*, **186**, 309 (1950).
2. London, I. M., Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **183**, 749 (1950).
3. Sakami, W., Evans, W. E., and Gurin, S. J., *J. Am. Chem. Soc.*, **69**, 1110 (1947).
4. Bouthillier, L. P., and D'Iorio, A., *Rev. canad. biol.*, **9**, 382 (1950).
5. Pyman, F. L., *J. Chem. Soc.*, **109**, 186 (1916).
6. Pyman, F. L., *J. Chem. Soc.*, **99**, 668, 1386 (1911).
7. Totter, J. R., and Darby, W. J., *Org. Syntheses*, **24**, 64 (1944).
8. Holleman, A. F., *Org. Syntheses*, coll. **1**, 497 (1932).

9. Marckwald, W., *Ber. chem. Ges.*, **29,** 42 (1896).
10. Read, J., and Reid, W. G., *J. Chem. Ind.*, **47,** 9T (1928).
11. Dubnoff, J. W., *Arch. Biochem.*, **17,** 327 (1948).
12. Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, **210,** 33 (1932).
13. Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H., *J. Biol. Chem.*, **179,** 689 (1949).
14. Fisher, R. A., Statistical methods for research workers, 2nd edition, Edinburgh (1928).
15. Borsook, H., Deasy, C. L., Haagen-Smith, A. J., Keighley, G., and Lowy, P. H., *J. Biol. Chem.*, **186,** 297 (1950).
16. Stein, W. H., and Moore, S., *Cold Spring Harbor Symposia Quant. Biol.*, **14,** 179 (1949).
17. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **178,** 53 (1949).
18. Melchior, J. B., Mellody, M., and Klotz, I. M., *J. Biol. Chem.*, **174,** 81 (1948).
19. Winnick, T., Moring-Claesson, I., and Greenberg, D. M., *J. Biol. Chem.*, **175,** 127 (1948).
20. Frantz, I. D., Jr., Loftfield, R. B., and Miller, W. W., *Science*, **106,** 544 (1947).
21. Johnston, R. B., and Bloch, K., *J. Biol. Chem.*, **188,** 221 (1951).
22. Drabkin, D. L., *J. Biol. Chem.*, **164,** 703 (1946).
23. Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141,** 627 (1941).
24. Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141,** 671 (1941).
25. Toennies, G., and Homiller, R. P., *J. Am. Chem. Soc.*, **64,** 3054 (1942).
26. Sanger, F., *Biochem. J.*, **39,** 507 (1945).
27. Herbst, R. M., and Shemin, D., *Org. Syntheses*, **2,** 11 (1944).
28. Porter, R. R., and Sanger, F., *Biochem. J.*, **42,** 287 (1948).
29. Schroeder, W. A., Kay, L. M., and Wells, I. C., *J. Biol. Chem.*, **187,** 221 (1950).
30. Moore, S., and Stein, W. H., *Ann. New York Acad. Sc.*, **49,** 265 (1948); *J. Biol. Chem.*, **176,** 367 (1948). Stein, W. H., and Moore, S., *J. Biol. Chem.*, **176,** 337 (1948); **178,** 79 (1949).
31. Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H., *Federation Proc.*, **8,** 589 (1949).
32. Mitchell, H. K., and Haskins, F. A., *Science*, **110,** 278 (1949).
33. Fontès, G., and Thivolle, L., *Compt. rend. Acad.*, **191,** 1088 (1930); *Le Sang*, **4,** 658 (1930).
34. Okugawa, Y., and Tatsui, M., *Z. physiol. Chem.*, **195,** 192 (1931).
35. Matsuoka, Z., and Nakao, T., *Z. physiol. Chem.*, **195,** 208 (1931).
36. Drabkin, D. L., and Miller, H. K., *J. Biol. Chem.*, **90,** 531 (1931); **93,** 39 (1931).
37. Elvehjem, C. A., Steenbock, H., and Hart, E. B., *J. Biol. Chem.*, **93,** 197 (1931).
38. Keil, H. L., and Nelson, V. E., *J. Biol. Chem.*, **97,** 115 (1932).
39. Alcock, R. S., *Biochem. J.*, **27,** 754 (1933).
40. Kotake, Y., *Ergeb. Physiol.*, **37,** 245 (1935).
41. Hamada, T., *Z. physiol. Chem.*, **243,** 258 (1936).
42. Pearson, P. B., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **119,** 749 (1937).
43. Chin Kyu-sui, *Z. physiol. Chem.*, **257,** 18 (1938–39).
44. Whipple, G. H., and Robscheit-Robbins, F. S., *Proc. Soc. Exp. Biol. and Med.*, **36,** 629 (1937); *J. Exp. Med.*, **71,** 569 (1940).
45. Harris, H. A., Neuberger, A., and Sanger, F., *Biochem. J.*, **37,** 508 (1943).
46. Albanese, A. A., Holt, L. E., Jr., Kajdi, C. N., and Frankston, J. E., *J. Biol. Chem.*, **148,** 299 (1943).

47. Gillespie, M., Neuberger, A., and Webster, T. A., *Biochem. J.*, **39,** 203 (1945).
48. Orten, A. U., and Orten, J. M., *J. Nutr.*, **30,** 137 (1945).
49. Kornberg, A., *Federation Proc.*, **5,** 142 (1946).
50. Yeshoda, K. M., and Damodaran, M., *Biochem. J.*, **41,** 382 (1947).
51. Daft, F. S., *Pub. Health Rep., U. S. P. H. S.*, **62,** 1785 (1947).
52. Orten, J. M., Bourque, J. E., and Orten, A. U., *J. Biol. Chem.*, **160,** 435 (1945).
53. Sebrell, H., *Federation Proc.*, **8,** 568 (1949).
54. Nizet, A., and Robscheit-Robbins, F. S., *Blood*, **5,** 648 (1950).
55. Chandran, K., and Damodaran, M., *Biochem. J.*, **49,** 393 (1951).