Incorporation of C^{14} From Carboxyl-labeled *dl*-Alanine Into the Proteins of Liver Slices[1]

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The source of energy for the formation of peptide bonds in living tissue is not known. Study of protein or peptide synthesis is hampered by the proteolysis which, in tissue outside the intact animal, always seems to predominate. Melchior and Tarver (*5*) have summarized the difficulties involved and have shown how they may be overcome by the use of tracers. The present paper describes preliminary studies of protein formation in liver slices, as measured by the incorporation of carboxyl-labeled alanine.

Carboxyl-labeled *dl*-alanine was prepared by the Strecker synthesis (*8*), with modifications appropriate for use of small amounts of radioactive hydrogen cyanide. The hydrogen cyanide was generated from potassium cyanide made by the method of Cramer and Kistiakowsky (*2*), with certain changes.

Eight-tenths gram of clean potassium was placed in a 10 mm. x 400 mm. Pyrex tube (capacity, about 14 cc.). This tube was attached to a vacuum line, and the potassium was shaken and distilled to form a continuous thin mirror over the entire inner surface of the tube. Approximately 2 millimoles of ammonia and the carbon dioxide from 8.5 mg. of radioactive barium carbonate (stated to contain 3–5 per cent $BaC^{14}O_3$) and from 179 mg. of nonradioactive barium carbonate were condensed in the reaction tube with liquid nitrogen. This tube, containing potassium, ammonia, and radioactive carbon dioxide, was sealed and placed in a steel bomb tube, which was inserted in an electric furnace. The temperature was raised to 620° C. in 8 minutes and was then held constant for 12 minutes. After cooling, the glass tube was removed and chilled in liquid nitrogen. The end of the tube was heated with a hot flame and the excess pressure released. The small amount of residual metal was decomposed with ethyl alcohol and water. After transfer to a generating flask on the vacuum line, and after preliminary evacuation, formic acid was added and the hydrogen cyanide distilled into a trap along with about 1 cc. of water. With inactive CO_2 the yield here was 90–96 per cent (average, 92 per cent).

The solution of hydrogen cyanide was transferred to a reaction flask along with 4 millimoles of ammonia and 2 millimoles of acetaldehyde and left overnight. Four cc. of 40 per cent hydrobromic acid was added and the solution boiled down to a paste during 4 hours. The paste was stirred at −40° C. with 3 cc. of methyl alcohol, filtered, and washed once with 2 cc. of cold methanol. The filtrate was neutralized with ammonium hydroxide. In order to recover the small amount of synthesized *dl*-alanine, 0.25 gram of ordinary *dl*-alanine was added, with heating. On cooling, 0.22 gram of carboxyl-labeled *dl*-alanine separated (m.p., 250° C. dec.; mixed m.p. with authentic pure sample, 250° C. dec.; activity 10 μc./mM).

Into Warburg flasks were pipetted 1–2 cc. of Krebs-Ringer phosphate solution (*3, 9*) and 0.1–0.2 cc. of a solution of radioactive *dl*-alanine containing 0.86 mg. of *dl*-alanine/cc. If 0.1 cc. was added, the total activity per flask was about 21,000 counts/minute. Two-tenths cc. of 10 per cent potassium hydroxide was placed in each center well.

An adult Wistar rat was killed by decapitation. The liver was removed, and slices about 0.5 mm. in thickness were cut. The dry weight of each slice was 10–20 mg. Two to four slices were placed in each flask, the flasks then being attached to their manometers and the air washed out with either oxygen or nitrogen. After incubation at 37° C., with shaking, for varying lengths of time, the slices were removed and rinsed with two 20-cc. portions of water. They were then placed in a test tube, with 5 cc. of water, and homogenized with a glass pestle (*7*). After transfer to a centrifuge tube, 5 cc. of 20 per cent trichloracetic acid was added. After 15 minutes, the precipitated proteins were centrifuged and washed three times with 10-cc. portions of 10 per cent trichloracetic acid. One cc. of 6 N hydrochloric acid was added. Hydrolysis of the proteins was effected by autoclaving for 16 hours at a pressure of 20 pounds. One drop of 0.1 per cent phenol red was added, and the hydrochloric acid was neutralized with concentrated ammonia. The volume was adjusted to about 2 cc. The carboxyl groups were then split from the amino acids by reaction with ninhydrin, according to the method recommended by Van Slyke, MacFadyen, and Hamilton (*10*). In this procedure, 50 mg. of powdered citrate buffer at pH 2.5 and 50 mg. of ninhydrin were used. The boiling time allowed was 15 minutes. The carbon dioxide was distilled into 2 cc. of approximately 0.25 N barium hydroxide, and the amount of barium carbonate formed was estimated by titration with 0.1 N hydrochloric acid. After the distillation system had been filled with carbon dioxide-free air, the tubes containing the barium carbonate were removed, stoppered, and set aside for counting.

Measurements of the activity in the carbon dioxide evolved in the ninhydrin reaction were carried out by transferring the gas sample directly into an evacuated counter tube. The gas-counting technique has been recently described (*6*). Such a method is desirable here because the low specific activity of the sample would reduce the efficiency of measurement by standard solid sample—end-window counter techniques to a low value.

The barium carbonate was rinsed into a gas evolution flask attached to the vacuum line. The carbon dioxide was evolved by addition of dilute perchloric acid, swept by a slow stream of nitrogen into a trap cooled in liquid nitrogen, then transferred to a gas burette for measurement of the amount of sample, and finally transferred by distillation to an evacuated counter tube attached to the gas system with ground joint and stopcock. The dose of carbon disulfide vapor necessary for counting was added and the counter tube then detached from the gas system and placed in a lead housing for counting.

A sufficient number of counts was collected in each sample to make the statistical error less than the other errors of tech-

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nique in the over-all procedure. The activities are reported as final corrected counting rates per minute in the samples (*6*).

The data from the first series of experiments are given in Table 1. It may be seen that the incorporation of activity into the trichloracetic acid precipitable protein is a slow and continuing process that is dependent on the utilization of oxygen by the tissue. The low values obtained in nitrogen and after very short incubation periods serve as a check on the efficiency of the washing procedure and demonstrate that this uptake is not a simple adsorption on the protein structure. Presumably because of individual differences between tissue slices, the scatter of the data accumulated to date does not allow the shape of the curve of uptake with time to be determined. It could be expected to go through a maximum and fall toward zero, because the net over-all process is proteolysis.

TABLE 1

No. of slices	Activity added (counts/ min.)	Vol. of liquid bathing slices (cc.)	Time	Gas phase	mM CO_2 derived from protein hydrolysate	Total counts/ min. incorporated into protein
2	21,000	1.1	1 min.	Air	0.103	12
2	21,000	1.1	1 "	"	0.121	13
2	21,000	1.1	4 hrs.	Nitrogen	0.124	12
2	21,000	1.1	4 "	"	0.091	4
2	21,000	1.1	4 "	"	0.146	0*
4	42,000	2.2	4 "	"	0.147	0
4	42,000	2.2	4 "	"	0.189	20*
2	21,000	1.1	2 "	Oxygen	0.115	102
2	21,000	1.1	2 "	"	0.131	150
2	21,000	1.1	2 "	"	0.112	126
2	21,000	1.1	4 "	"	0.144	916†
2	21,000	1.1	4 "	"	0.125	160
2	21,000	1.1	4 "	"	0.102	166
2	21,000	1.1	4 "	"	0.146	76*
4	42,000	2.2	4 "	"	0.153	188
4	42,000	2.2	4 "	"	0.146	128*

* The pH of the liquid phase in these experiments was 6.96; that in all other experiments, approximately 7.25.

† This value is thought to be greatly in error. It is included in the interest of completeness.

Because of the variety of reactions in which alanine may participate, detection of activity in protein does not prove that alanine molecules, as such, have entered the peptide chain. Preliminary experiments, in which relatively large quantities of nonradioactive *dl*-alanine were added to a tagged hydrolysate and the alanine crystallized three times, do indicate that a large percentage of the activity in the hydrolysate can be accounted for as alanine. Small amounts of activity were found when similar crystallizations were conducted with *l*(+)-glutamic acid and with *dl*-aspartic acid. Whether this activity represents contamination with alanine cannot yet be stated. It would be expected, in the light of the recent work of Anfinsen, *et al.* (*1*) on the incorporation of activity from bicarbonate ion into the glutamic and aspartic acid fraction of protein, that a similar path to these two species would be available by way of pyruvic acid derived from alanine. No activity was found in glycine, the only other fraction so far investigated.

We regard as slight the possibility that activity has been incorporated by transcarboxylation with the original peptide chain, particularly in view of the reappearance of the activity in the alanine fraction. However, this possibility will be investigated with amino acids tagged elsewhere than in the carboxyl group.

Although the pathway by which alanine is incorporated into the proteins of liver slices remains obscure, it seems clear that under the present experimental conditions little or no incorporation is possible in the absence of oxygen. All experiments completed to date confirm this observation. This finding is compatible with theories of protein synthesis which postulate coupling with energy yielding reactions (cf. Lipmann, *4*).

References

1. Anfinsen, C. B., Beloff, A., Hastings, A. B., and Solomon, A. K. *J. biol. Chem.*, 1947, **168**, 771.
2. Cramer, R. D., and Kistiakowsky, G. B. *J. biol. Chem.*, 1941, **137**, 549.
3. Krebs, H. A., and Henseleit, K. *Z. physiol. Chem.*, 1932, **210**, 33.
4. Lipmann, F. *In* Nord, F. F., and Werkman, C. H. *Advances in enzymology and related subjects.* (Vol. 1.) New York: Interscience, 1941. P. 99
5. Melchior, J. B., and Tarver, H. *Arch. Biochem.*, 1947, **12**, 301, 309.
6. Miller, W. W. *Science*, 1947, **105**, 123.
7. Potter, V. R., and Elvehjem, C. A. *J. biol. Chem.*, 1936, **114**, 495.
8. Strecker, A. *Ann. Chem.*, 1850, **75**, 27.
9. Umbreit, W. W., Burris, R. H., and Stauffer, J. F. *Manometric techniques and related methods for the study of tissue metabolism.* Minneapolis: Burgess, 1945. P. 194.
10. Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P. *J. biol. Chem.*, 1941, **141**, 671.

Isolation of Avian Pneumoencephalitis (Newcastle Disease) Virus From the Yolk Sac of Four-Day-Old Chicks, Embryos, and Infertile Eggs

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Beach (*1*) has reported the isolation of pneumoencephalitis virus from ovarian tissue of a hen, while Jungherr (*2*) and Van Roekel (*3*) have obtained the virus from fresh eggs. These findings show that the virus may be present in hatching eggs but do not provide evidence that it will survive the incubation period and result in chicks being infected when hatched.

In the experiments reported herein, the four-day-old chicks, the embryos, and the infertile eggs used for virus isolation trials were from parent stock known to be affected with pneumoencephalitis. The chicks were hatched from eggs obtained when egg production was severely depressed as a result of pneumoencephalitis. The embryos, from the same parent stock as the chicks, were from eggs collected when the hens were in the recovery period and the rate of egg production was returning to normal. The infertile eggs, from a second flock of breeders, were collected immediately prior to observed clinical evidence of infection, during the outbreak and through the recovery period.

The virus of pneumoencephalitis was isolated by chick embryo culture from the yolk-sac content of 6 four-day-old chicks and from embryos of the succeeding hatch dead on the 15th day of incubation.

Fifty eggs from the second breeding flock were incubated at this laboratory. The virus was isolated from the pooled con-