SYNTHESIS OF PROTEIN AND OTHER CELL SUBSTANCES FROM ACETIC ACID IN ISOLATED BONE MARROW*

by

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It is now generally accepted that the oxidative degradation of various foodstuffs to carbon dioxide and water involves a two-carbon intermediate related to acetic acid. It is not surprising, then, that acetic acid should in turn serve as a building block for many more complex molecules. Indeed, BLOCH, in his excellent review[1] on the metabolism of acetic acid in animal tissues, has discussed the participation of acetate in a large number of synthetic processes. Incorporation of acetate into the protein molecule was demonstrated by RITTENBERG AND BLOCH[2] by feeding C^{13} labelled acetate to rats and mice. Glutamic and aspartic acids and arginine isolated from the liver proteins contained excess C^{13}. Quite recently, GREENBERG AND WINNICK[3] have repeated these experiments and confirmed them. *In vivo* experiments, however, are limited by the fact that the composition of the medium and the role of other tissues cannot be controlled. In order to study the role of acetate in the synthesis of proteins it was therefore necessary to find an animal tissue where such reactions could be made to proceed *in vitro* and in a relatively short time.

Of all the tissues of the body of an adult animal, the bone marrow is the most active in the formation of new cells. Furthermore, acetate is readily utilized by this tissue. It was decided for these reasons to choose the bone marrow for a study of the role of acetate in processes of synthesis. Using carboxyl labelled acetate, it was found that C^{14} was rapidly incorporated into protein, phospholipid, and fatty acids, and more slowly into nucleic acids. The distribution of C^{14} in the α,γ carboxyl groups of glutamic acid and the α,β carboxyl groups of aspartic acid in the protein, and the necessity of the respiratory process for protein synthesis find clear explanation in the experiments of RUDOLPH AND BARRON[5] who have demonstrated that in kidney homogenates citric acid is formed by the condensation of acetate with oxaloacetate. In the oxidative degradation of citric acid, α-ketoglutaric acid and oxaloacetic acid are transformed by amination to glutamic acid and aspartic acid.

These experiments were presented at the 1948 meetings of the Society of General Physiologists[6]. Incorporation of C^{14} from $NaHC^{14}O_3$ and $CH_3C^{14}OONa$ into protein in *in vitro* experiments with rabbit liver has recently been reported by ANFINSEN *et al*[7].

METHODS

Preparation of Tissues. Young adult rabbits weighing about 2 kg were used in these experiments.

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The animals were bled by carotid section, and the humeri, fibulae and femora were removed. The cortex was peeled off using pointed rongeurs; the marrow sausages were freed by blunt dissection; and the specimens were chilled in an ice-cold Petri dish. Slices were prepared free hand with a thin razor blade and were kept in ice-cold Ringer-phosphate until the beginning of the experimental period. Homogenates were prepared with a loose glass homogenizer.

Acetate Analysis. Acetate was determined by the method of FRIEDEMAN[8], and then separated from the steam distillate for C^{14} analysis by the following procedure. After titration, the neutral solutions were diluted (usually 1 : 200) with unlabelled acetic acid and were made alkaline to phenolphthalein. The volume was then reduced by boiling to 5 to 10 ml and made acid with dilute HNO_3. The solution was brought to p_H 6.2 with dilute NH_4OH (using brom cresol purple as an indicator). Acetate was precipitated as silver acetate by the addition of an excess of $AgNO_3$. The solution containing the precipitated silver acetate was heated to boiling and filtered hot through a sintered glass filter. The silver acetate crystallized out on cooling and was filtered and washed with small amounts of H_2O, and dried in a dessicator. The silver salts were combusted as described below for radioactivity assay.

Measurement of Radioactivity. Specific activity (S.A.) as used in this paper is a measure of the *relative concentration* of C^{14} atoms in the C^{12} matrix, and is defined as observed counts per minute when the sample, as $BaCO_3$, is packed in an infinitely thick layer (20 mg per cm^2) in an aluminum dish (area = 1.41 cm^2) and counted with a thin window counter in a standard geometry (27% of 4π radians). All organic samples were dry combusted to CO_2 (in a micro-combustion tube with Pt gauze at 800°) which was bubbled through saturated $Ba(OH)_2$, and the precipitated $BaCO_3$ was washed repeatedly with water and methanol and dried in the aluminum dishes ready for counting.

Fractionation of Tissue. At the end of the experiments the tissue (from 2 or 3 rabbits) was centrifuged off; cold absolute ethanol (100 ml) was added and the tissue was ground in a WARING blendor for 15 min. After centrifugation, the marrow was resuspended in 200 ml of ethanol-ether (3 : 1) and was boiled for 2 hours. The solid fraction was filtered, washed with ether, and dried. The solvent extracts were set aside as the lipid fraction. Nucleic acids were extracted from this dry material by the method of HAMMARSTEN[9], and any remaining nucleotides or acid soluble components were removed by extraction with 100 ml of 7% trichloracetic acid at 90° for 15 minutes. The solid residue was centrifuged down, was washed with alcohol and ether, and dried as the protein fraction. The ninhydrin method of VAN SLYKE *et al.*[10] was used to liberate α-carboxyl groups after hydrolysis (30 hours in 6 *N* HCl).

Glutamic and aspartic acids were separated from the protein hydrolysate by the use of carriers (approximately 400 mg of L-glutamic and of L-aspartic). The glutamic acid was recrystallized 4 times from water, then converted to the hydrochloride and crystallized twice from concentrated HCl. Aspartic acid was twice recrystallized from water, then converted to the cupric salt which was recrystallized 3 times from water. Finally the copper was removed with H_2S and the free acid crystallized from alcohol-water. Succinic acid was also isolated by the carrier technique. After the addition of 400 mg of succinic acid, the residue from the steam distillation was extracted for 6 hours in the KEUTCHER STEUDEL extractor. Succinic acid was recrystallized once from hot water and was converted into the silver salt. This was reprecipitated five times to a constant specific activity. Both carboxyl groups were removed by the slow addition of a carbon tetrachloride solution of bromine[11], and the resulting CO_2 was trapped as $BaCO_3$.

For the isolation of phospholipids and saturated fatty acids, the alcohol-ether extract of the marrow was evaporated to dryness under reduced pressure at 30°. The lipid residue was dissolved in moist ether and the combined phospholipid was precipitated by the addition of 8 to 10 volumes of acetone. The precipitate, after washing 2 to 3 times with acetone, was redissolved in moist ether. In some of the experiments an attempt was made to isolate cephalin by absolute alcohol precipitation from ether, but none was found, and in subsequent experiments only the acetone precipitations were used. The "lecithin" was reprecipitated from ether 3 to 5 times, dry combusted, and counted as $BaCO_3$. The small quantities of material made degradation of the molecule impossible. The ether-acetone supernatant was evaporated to dryness and the lipid residue was saponified with KOH. Unsaponified material was removed by ether extraction from alkaline solution, and the free fatty acids were salted out of the acidified water layer. The mixed free fatty acids were then dissolved in acetone and the saturated higher fatty acids were precipitated by lowering the temperature to −30°. This material was recrystallized three times to a constant specific activity.

RESULTS

FORMATION OF PROTEIN FROM LABELLED ACETATE

Utilization of $CH_3C^{14}OONa$ and $NaHC^{14}O_3$ by Bone Marrow Slices. The first experiments on the synthesis of protein from acetate as one of the building materials were

conducted under conditions of optimum *in vitro* metabolism for this tissue. The incubation was carried out at 38° in an atmosphere of O_2 : CO_2 (95 : 5) in Ringer-bicarbonate containing acetate and glycine (0.005 *M*). The pooled slices were introduced into a battery of intercommunicating pyrex glass vessels through which the gas mixture was bubbled continuously to prevent reincorporation of labelled respiratory CO_2 into the protein. The time of incubation was six hours. In the experiments with bicarbonate labelled with C^{14}, the vessels were previously saturated with O_2; the gas phase was O_2; and the buffer was phosphate (0.01 *M*) of p_H 7.4. The vessels were kept closed during the entire experiment.

The results of three such experiments are shown in Table I.

TABLE I

INCORPORATION OF C^{14} INTO PROTEIN OF BONE MARROW AFTER INCUBATING SLICES FOR 6 HOURS WITH $CH_3C^{14}OONa$ AND $NaHC^{14}O_3$

Experiment No.	1		2		3	
Substrate:	0.01 *M* $CH_3C^{14}OONa$		0.004 *M* $CH_3C^{14}OONa$		0.01 *M* $NaHC^{14}O_3$	
	S.A.*	R.S.A.**	S.A.	R.S.A.	S.A.	R.S.A.
Substrate	85 000	100	143 000	100	162 000	100
Protein-total	205	0.24	—	—	31	0.02
Protein-α-COOH***	520	0.61	930	0.65	133	0.08

* Specific activity is measured in counts per minute at infinite thickness as defined in the section on measurement.

** Relative specific activity is based upon the substrate in the medium taken as 100.

*** Carbon dioxide which is liberated when the protein hydrolysate is treated with ninhydrin includes the β-carboxyl group of aspartic acid in addition to the α-carboxyl groups.

It is evident that acetate carboxyl is incorporated in the protein of isolated bone marrow. And this *in vitro* synthesis takes place to a greater extent in 6 hours than was found *in vivo* in rat liver in 3 days (2) or rat intestine in 8 hours (3). Furthermore, the fact that CO_2 is not an intermediate in this process is demonstrated by Exp. 3. Here the rate of incorporation of CO_2 is much lower than that of acetate, and the ratio of carboxyl carbon to total carbon is much lower with acetate than with carbonate (2.6 as compared to 4.2), indicating that in the case of acetate a larger proportion of the C^{14} is not liberated by ninhydrin.

Location of Acetate Carboxyl Carbon in Protein. To study the distribution of C^{14} in the different groups of the isolated protein, the experiments were performed in Ringer-phosphate buffer with O_2 as gas phase. The added substrates were C^{13}-carboxyl labelled glycine, C^{14}-carboxyl labelled acetate, and glutamate (in some experiments). Because it seemed most probable that acetate would be utilized via the tricarboxylic acid cycle, effort was concentrated on glutamic and aspartic acids (presumably formed from α-keto-glutaric and oxaloacetic acids).

The marrow slices were incubated for five hours. Following incubation, sulfuric acid and sodium tungstate were added to precipitate the protein. After the addition of carriers, glutamic and succinic acids were isolated from the non-protein filtrate, and glutamic and aspartic acids from the protein hydrolysate.

The results of the experiments are shown in Tables II and III. Acetate was rapidly

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TABLE II

DISTRIBUTION OF C^{14} AFTER 5 HOUR INCUBATION OF BONE MARROW SLICES WITH $CH_3C^{14}OONa$

Experiment No.	7		8	
Substrates:				
Glutamate, *M*	—		0.01	
Glycine, *M*	0.01		0.01	
Acetate, *M*	0.01		0.01	
	S.A.	Percent* of Added C^{14}	S.A.	Percent of Added C^{14}
Acetate	377 000	100	377 000	100
Protein:				
— Total	292	0.65	326	0.70
— α-Carboxyl	564	0.27	685	0.39
Non-Protein Glutamate: (370 mg carrier)				
— Total	66	0.47	144	1.03
— α-Carboxyl	90	0.13	198	0.28
Succinic Acid: (400 mg carrier)				
— Total	33	0.26	—	—
— Carboxyl	66	0.26	—	—

* Total C^{14} in each fraction was determined by multiplying the specific activity by the total millimoles of carbon in the fraction.

TABLE III

DISTRIBUTION OF ACETATE CARBOXYL CARBON IN BONE MARROW PROTEIN

Experiment No.	7	8
Fraction	Percent of total C^{14} in protein	
Protein: Total	100	100
Protein: α-Carboxyl	43	57
Glutamic Acid: Total	47	55
Glutamic Acid: α-Carboxyl	12	14
Aspartic Acid: Total	14	14
Aspartic Acid: α,β-Carboxyl	14	14

incorporated into the bone marrow protein. Evidence that it first went into glutamic acid, which was then incorporated into the protein, is given by the relatively large amount in the free glutamate fraction and by the increased amount of C^{14} in the free glutamate when the steady state of glutamate concentration was increased. However, there was no increase in incorporation into the protein. Added glutamate had no effect upon the rate of protein synthesis as measured with labelled glycine (glycine substrate, 51.2 atom % excess C^{13} in the carboxyl group; ninhydrin-liberated CO_2 of protein hydrolysate without added glutamate 0.10 atom % excess C^{13}; with added glutamate, 0.11 atom % excess C^{13}). The implication that the free glutamate had the same specific activity in both experiments is the expected result if one assumes a rapid equilibrium

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between α-ketoglutarate and glutamate. The fact that a relatively small fraction of the C^{14} in glutamic acid is in the α-carboxyl group, is in agreement with the hypothesis that acetate is utilized via the tricarboxylic acid cycle to form α-ketoglutarate (and glutamate) in which the γ-carboxyl group is labelled. Since all of the C^{14} of the protein aspartic acid and of the succinic acid was in the carboxyl groups, the remainder of the glutamate C^{14} was most certainly in the γ-carboxyl group. Essentially all of the protein C^{14} not present in α-carboxyl groups (including the β-carboxyl of aspartate) may be accounted for as γ-carboxyl of glutamate.

The Effect of Cell Damage and of Anaerobiosis on Protein Synthesis from Acetate. These experiments were performed in the presence of Ringer-phosphate containing $CH_3C^{14}OONa$, and boiled yeast juice. Cell damage was produced by grinding the bone marrow in a glass homogenizer. Since, under these conditions, respiration in the homogenates falls off rapidly, the metabolic processes were stopped after incubation times of 1, 1.5, 3, and 5 hours by the addition of H_2SO_4. In the aerobic experiments, the gas phase was oxygen. In the anaerobic experiments, the gas phase was N_2 : CO_2 (95 : 5). The results of these experiments are shown in Fig. 1 and 2. It can be seen that the rate of protein turnover remains constant as long as the respiration remains steady. In the homogenate, where the rate of respiration decreased continuously, the incorporation of acetate carbon into protein also decreased. In the absence of oxygen there was no utilization of the acetate carboxyl group for protein formation.

Formation of Phospholipids and Satured Fatty Acids from Acetate. In these experiments, the lecithin and saturated fatty acids were isolated from the ether-alcohol extracts of the preceding experiments. In all cases there was appreciable incorporation

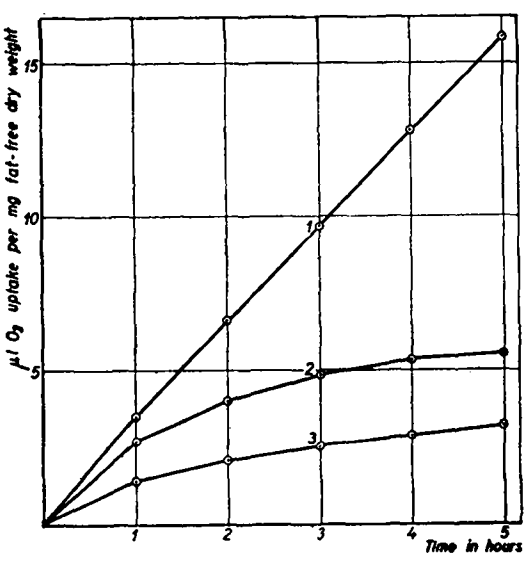

Fig. 1. Metabolic Activity of Bone Marrow Preparations. Abscissa, time in hours. Ordinate, μl O_2 uptake per mg fat-free dry weight.

1. O_2 uptake, slices in O_2 atmosphere.
2. O_2 uptake, homogenate in O_2 atmosphere.
3. CO_2 production, homogenate in N_2 atmosphere

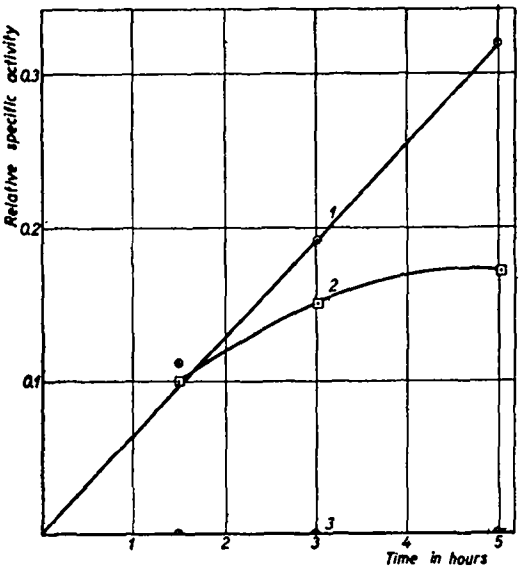

Fig. 2. Rate of Incorporation of the Labelled Carboxyl Group of Acetate in the Protein of Bone Marrow. Abscissa, time in hours. Ordinate, relative specific activity (as defined in the text).

1. Slices in O_2 atmosphere.
2. Homogenate in O_2 atmosphere
3. Homogenate in N_2 atmosphere.

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of C^{14} into these substances. The fact that the rate of turnover of phospholipid was markedly greater than that of saturated fatty acids, as indicated in Table IV, may result from the relatively great dilution of the fatty acids by inactive stored fat in the

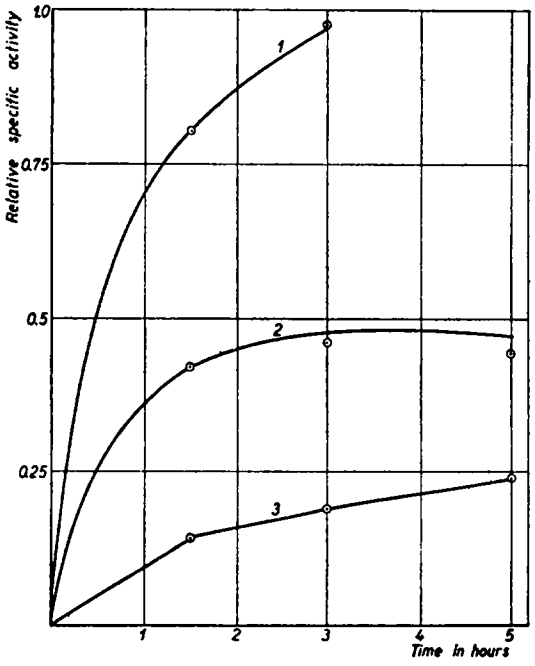

Fig. 3. Rate of Incorporation of the Labelled Carboxyl Group of Acetate in the Lecithin Fraction of Bone Marrow. Abscissa, time in hours. Ordinate, relative specific activity.

1. Slices in O_2 atmosphere.
2. Homogenate in O_2 atmosphere.
3. Homogenate in N_2 atmosphere.

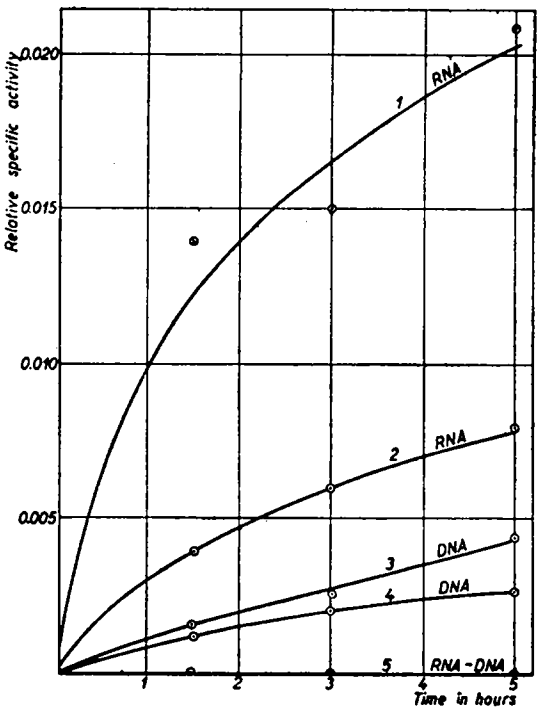

Fig. 4. Formation of Nucleic Acids from Carboxyl Labelled Acetate in Isolated Bone Marrow. Abscissa, time in hours. Ordinate, relative specific activity.

1. Ribonucleic acid (RNA) formation in slices in oxygen.
2. RNA in marrow homogenate in oxygen.
3. Desoxyribonucleic acid (DNA) in slices in oxygen.
4. DNA in homogenate in oxygen.
5. RNA and DNA in homogenates in nitrogen.

TABLE IV

INCORPORATION OF C^{14} INTO PHOSPHOLIPID AND FATTY ACIDS AFTER INCUBATING BONE MARROW SLICES FOR 6 HOURS WITH $CH_3C^{14}OONa$ AND $NaHC^{14}O_3$

Experiment No.	2		3	
Substrate:	0.004 *M* $CH_3C^{14}OONa$		0.01 *M* $NaHC^{14}O_3$	
	S.A.	R.S.A.	S.A.	R.S.A.
Labelled Substrate	143 000	100	162 000	100
Phospholipid	1 290	0.90	113	0.07
Saturated Fatty Acids	140	0.10	4	0.00

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marrow. While protein synthesis stopped in the absence of respiration, a small amount of phospholipid synthesis proceeded under anaerobic conditions, as shown in Fig. 3. The possibility of contamination by labelled acetic acid was ruled out by control experiments. The incorporation of bicarbonate carbon into phospholipid was only a small fraction of that from acetate carboxyl carbon. There was essentially no bicarbonate C^{14} found in the saturated fatty acid fraction.

Formation of Ribonucleic and Desoxyribonucleic Acids from Acetate. Ribonucleic and desoxyribonucleic acids were isolated in most of these experiments. The results of three such experiments are given in Fig. 4. The nucleic acid activities were low, contrary to our expectations for a tissue actively synthesizing protein. As is characteristic of all mammalian tissues so far investigated, the desoxyribonucleic acid was initially renewed at a slower rate than the ribonucleic acid, although the ribonucleic acid rate fell more rapidly with time. There was no strict parallelism between the rate of incorporation of acetate into ribonucleic acid and into protein. Again homogenization caused a decrease in the rate of turnover, and there was no turnover under anaerobic conditions. The rates of incorporation from $NaHC^{14}O_3$ were similar. The amounts of nucleic acid isolated in these experiments were insufficient for degradation.

SUMMARY

Carboxyl labelled acetic acid has been used to study the synthetic processes occurring in isolated bone marrow—slices and homogenates. The labelled carbon was rapidly incorporated into protein and phospholipid, more slowly into nucleic acids. The rate of protein synthesis parallelled the respiratory rate. All of the C^{14} in the protein was in carboxyl groups, with the major site being the carboxyl groups of glutamic and aspartic acids— particularly the γ-carboxyl group of glutamic acid.

RÉSUMÉ

Nous avons employé de l'acide acétique marqué au groupe carboxyl pour étudier les processus de synthèse ayant lieu dans la moëlle osseuse isolée — en tranches ou en homogénats. Le carbone marqué était incorporé rapidement dans les protéines et les phospholipoïdes, plus lentement dans les acides nucléiques. La synthèse des protéines allait parallèlement avec la respiration. Tous le C^{14} contenu dans les protéines se trouvait dans les groupes carboxyl, en majeure partie dans les groupes carboxyl des acides glutamique et aspartique et en particulier dans le groupe γ-carboxyl de l'acide glutamique.

ZUSAMMENFASSUNG

An der Carboxylgruppe markierte Essigsäure wurde zur Untersuchung der synthetischen Vorgänge im isolierten Knochenmark — in Scheiben und in Homogenaten — verwendet. Der markierte Kohlenstoff wurde rasch in Eiweisskörper und Phospholipoïde, langsamer in Nukleinsäuren einverleibt. Die Proteinsynthese verlief parallel mit der Atmung. Die Gesamtheit des C^{14}-Kohlenstoffes im Eiweiss fand sich in den Carboxylgruppen, hauptsächlich in den Carboxylgruppen der Glutamin- und Asparaginsäure, insbesondere in der γ-Carboxylgruppe der Glutaminsäure.

REFERENCES

1 K. BLOCH, *Physiol. Revs*, 27 (1947) 574.
2 D. RITTENBERG AND K. BLOCH, *J. Biol. Chem.*, 157 (1945) 749.
3 D. M. GREENBERG AND T. WINNICK, *Arch. Biochem.*, 21 (1949) 166.
4 J. M. GOLDINGER, M. A. LIPTON, AND E. S. G. BARRON, *J. Biol. Chem.*, 171 (1947) 801.
5 G. RUDOLPH AND E. S. G. BARRON, *Biochim. Biophys. Acta*, 5 (1950) 59.
6 R. ABRAMS, J. M. GOLDINGER, AND E. S. G. BARRON, *Biol. Bull.*, 95 (1948) 284.
7 C. B. ANFINSEN, A. BELOFF, AND A. K. SOLOMON, *J. Biol. Chem.*, 179 (1949) 1001.
8 T. FRIEDEMAN, *J. Biol. Chem.*, 123 (1938) 161.
9 E. HAMMARSTEN, *Acta Med. Scand., Suppl.* 196 (1947) 634.
10 D. D. VAN SLYKE, R. T. DILLON, D. A. MACFAYDEN, AND P. HAMILTON, *J. Biol. Chem.*, 141 (1941) 627.
11 H. HUNSDIECKER AND C. HUNSDIECKER, *Ber.*, 75 (1942) 291.

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