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Enzymatic Hydrolysis and Synthesis of Benzohydroxamic Acid

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The participation of hydroxylamine in the nitrogen metabolism has been difficult to interpret chiefly because no enzymes have been known to act, *e. g.* in the formation of oximes via reactions between hydroxylamine and oxo-compounds. True, the spontaneous reaction of certain α-keto acids and aldehyde acids with hydroxylamine is so rapid that hydroxylamine, formed in the organisms at the natural concentrations of these acids, may be continuously removed without any catalysts via oxime formation. Of all the examined α-oxo-acids, glyoxylic, pyruvic, oxaloacetic, and ketoglutaric acids, the oximes of which correspond as reduction products to glycine, α-alanine, aspartic and glutamic acids, react according to Virtanen and Alfthan [1] most readily with hydroxylamine in the given order. In the synthesis of amino acids *in vivo* one would expect, however, a certain guidance of reactions by specific enzymes, and hence, a control of chemical reactions.

The first information found in the literature of the participation of hydroxylamine in an enzymatic reaction is the observation of Jacobsohn and Soares [2] on the linkage of hydroxylamine with the double bond of fumaric acid by the effect of aspartase or an analogous enzyme. We have, however, in this laboratory failed to confirm this reaction. Speck [3] and Elliott [4] proved convincingly two years ago that in the enzymatic synthesis of glutamine from glutamic acid and ammonia, the latter is replacable by hydroxylamine whereby hydroxamic acid is formed:

$$\text{Glutamate} + NH_3(NH_2OH) \rightarrow \text{glutamine (glutamohydroxamic acid)} \qquad (1)$$

Waelsch *et al.*[5, 6], Grossowicz *et al.*[7] found recently in certain microorganisms an enzyme, which causes the following reaction:

$$\text{Glutamine (asparagine)} + NH_2OH \rightarrow \text{glutamohydroxamic (aspartohydroxamic) acid} + NH_3 \qquad (2)$$

The experiments with $N^{15}H_3$ showed that the same enzyme extract also catalyzes the exchange of the amide group of glutamine and asparagine with ammonia. Reaction (1) requires utilization of adenosinetriphosphate [3,4] as an external source of energy, reaction (2) proceeds without utilization of energy-rich phosphate bonds [5–7]. Mg^{++} ions are necessary for the reaction (1) which is strongly depressed by sodium fluoride in low concentrations. Neither of these has any influence on the reaction (2).

Stumpf and Loomis [8,9] reported having found in pumpkin seedlings an enzyme (glutamyltransphorase), which labilizes the γ-amide group of glutamine and thus catalyzes the formation of glutamohydroxamic acid from glutamine and hydroxylamine. This enzyme is according to Stumpf (personal communication) very widely spread in the plant kingdom, *e. g.* it is liberally found in the root nodules of lupin and clover as well as in *Azotobacter agilis*. Contrary to the finding of Waelsch *et al.* ATP or ADP is indispensable to the activity of the enzyme of Stumpf and Loomis. Moreover, this enzyme does not act when asparagine replaces glutamine, although a raw preparation of pumpkin seedlings causes a reaction between hydroxylamine and asparagine. Manganese and phosphate or arsenate belong to the complete enzyme system of Stumpf and Loomis, fluoride causes complete inhibition.

We have been interested in finding out whether the activity of the enzyme system catalyzing the above reactions is restricted in glutamic acid, glutamine and asparagine or whether the activity is of more general nature. Therefore, we have examined the enzymatic synthesis and hydrolysis of benzohydroxamic acid and benzamide and report our results in the following. Speck has found a number of carboxyl acids ineffective in the reaction (1) and Grossowicz *et al.* some amides (eg. benzamide) in the reaction (2).

The possible significance of the active amide group of glutamine at the amination reactions has already earlier attracted attention [10, 11] and it might not be impossible that analogously the $>$ NOH-group could be transferred from hydroxamic acid. The formation of hydroxamic acids might also have another function, viz. the reduction of hydroxylamine to ammonia. This would require a reducing system for the $>$ NOH group of hydroxamic acids. If such a reducing system were found in the cells, this explanation would be the most plausible one for the function of hydroxamic acids in the nitrogen metabolism.

EXPERIMENTAL

The substrates and enzyme preparations

Benzamide was prepared according to Fischer [12] from ammonium carbonate and benzoyl chloride; m. p. 126°.

Benzohydroxamic acid was prepared according to Renfrow and Hauser [13] from hydroxylamine and ethylbenzoate. The m.p. of our preparation was 123°.

Yeast autolysate was prepared chiefly according to Grassmann and Mayr [14]. 100 g of baker's yeast was mixed with 10 ml of toluene for an hour whereby the yeast was liquefied. Water was added up to 100 ml and the suspension was centrifuged. The residue was diluted with water to 100 ml, allowed to stand for 15—20 h and centrifuged. The solution thus obtained (yeast-autolysate) decomposes benzamide and benzohydroxamic acid. The baker's yeast was a product of Rajamäki factories (Hyvinkää). The experiments were carried out in summer 1950. As later at the end of the same year the yeast from the same factory was autolyzed, the autolysate was inactive. The factory informed having changed the yeast strain in the meanwhile. The activity of the different yeast strains was not examined in detail, it was merely observed that the laboratory transfer of *Torula utilis* did not produce an active autolysate. Nor could such one be prepared from the brewer's yeast.

Sheep-liver extract. 500 g of liver from a just slaughtered sheep was rapidly cut into pieces, ground in a meat grinder and rubbed with sand. 750 ml of water were added, kept in an ice-cupboard for an hour, and centrifuged. The supernatant liquid was used in the experiments either immediately or after dialysis. Bray *et al.*[15] have employed a similar method when preparing an extract from the liver and kidneys of hen for examination of the hydrolysis of amides.

Analytical methods

Hydroxamic acid was quantitatively determined according to Lipmann and Tuttle [16]. Benzohydroxamic acid served as a standard also for glutamohydroxamic acid since a suitable standard for that was unavailable. The intensity of the colour was determined by a Klett-Summerson photometer using filter S 54. In the latest experiments a slightly modified method of Stumpf (personal communication) was used with filter S 50.

Ammonia was determined according to Pucher.

Hydroxylamine was determined according to the method of Blom as modified by Csáky [17].

Phosphorus was determined according to Berenblum and Chain [18].

Benzoic acid was identified with a paperchromatographic method. Filter paper Whatman no. 1 was used and as a solvent butylalcohol with a drop of ethylamine. After 4 h the paper was dried and sprayed with bromchresolgreen. A distinct blue spot was detectable at about 5 cm distance from the initial drop. The spot was not fluorescent in the ultraviolet light.

In another method the paper was treated at first with 0.1 % fluorescein solution. A drop of benzoic acid solution produces a spot on the paper, which in the ultraviolet light is dark and not fluorescent. Benzohydroxamic acid and benzamide produce a spot which is light and fluorescent in the ultraviolet light. A mixture of benzohydroxamic acid and benzoic acid gives a spot which is similar to that given by benzoic acid.

Experiments and results

Hydrolysis

Experiments were made with both enzyme preparations in order to find out whether yeast autolysate and liver extract, which according to different authors, *e. g.* Gonnermann [19] (liver) Geddes and Hunter [20] (yeast), Grassmann and Mayr [14] (yeast), and Bray *et al.*[15] (liver) hydrolyze different amides, are able to split off hydroxylamine from benzohydroxamic acid.

A. *Experiment with yeast autolysate.* Benzohydroxamic acid (BHA) was dissolved in a phosphate buffer with pH 7.3 (0.002 M BHA-solution).

I 25 ml of BHA-solution + 5 ml of yeast autolysate
II 25 ml of BHA-solution + 5 ml of boiled yeast autolysate
III 25 ml of buffer solution + 5 ml of yeast autolysate
IV 25 ml of BHA-solution + 5 ml of water.

B. *Experiment with liver extract.* Benzohydroxamic acid was dissolved in

I. phosphate buffer with pH 7.3 (0.002 M BHA-solution).
II. glycine-NaOH buffer with pH 7.4 (0.002 M BHA-solution).
a. 25 ml of BHA-solution + 5 ml of liver extract
b. 25 ml of BHA-solution + 5 ml of boiled liver extract
c. 25 ml of buffer solution + 5 ml of liver extract.

Samples of 1 ml were taken at definite intervals from each experiment. E-values from the BHA-determinations are presented in Table 1.

Table 1. E-values from the BHA-determinations.

A. Yeast autolysate					B. Liver extract						
Time h	I	II	III	IV	Time h	Ia	Ib	Ic	IIa	IIb	IIc
0	128	130	11	143	0	148	142	15	165	155	16
3	78	130	11	143	2	110	140	7	135	154	12
					4	91	142	5	121	154	5
					7	69	139	6	116	154	5
19	0	130	11	143	17	60	141	5	103	154	5

The yeast autolysate (expt. A) hydrolyzed in 3 h 39 % of benzohydroxamic acid and in 19 h the hydrolysis was complete; the liver extract (expt. B) hydrolyzed in 2 h 25.6 % and in 17 ½ h 59.5 % of hydroxamic acid in the phosphate solution and in the glycine solution respectively 18.2 % and 37.6 %.

The hydrolysis of benzamide with the same enzyme preparations is illustrated by the following experiments.

A. *Experiment with yeast autolysate.* Benzamide (BA) was dissolved in a phosphate buffer with pH 7.3 (0.0174 M BHA-solution).

I 25 ml of BA-solution + 5 ml of yeast autolysate
II 25 ml of BA-solution + 5 ml of boiled yeast autolysate
III 25 ml of buffer solution + 5 ml of yeast autolysate
IV 25 ml of BA-solution + 5 ml of water

B. *Experiment with liver extract.* Benzamide (BA) was dissolved in

I. phosphate buffer with pH 7.3 (0.0174 mol BA-solution)
II. glycine-phosphate buffer with pH 7.4 (0.0174 M BA-solution)
a. 25 ml of BA-solution + 5 ml of liver extract
b. 25 ml of BA-solution + 5 ml of boiled liver extract
c. 25 ml of buffer solution + 5 ml of liver extract.

Samples of 5 ml were used for NH_3-determinations. NH_3 found in the solutions, is to be seen in Table 2.

Table 2. NH_3 found in the solutions, mg.

A. Yeast autolysate					B. Liver extract						
Time h	I	II	III	IV	Time h	Ia	Ib	Ic	IIa	IIb	IIc
0	0.204	0.204	0.202	0.030	0	0.048	0.039	0.046	0.048	0.041	0.041
4	—	—	—	—	4	0.105	0.043	0.046	0.076	0.043	0.043
17 ½	0.549	0.197	0.172	0.034	17 ½	0.156	—	—	0.117	—	—

Within 17 ½ h the yeast autolysate hydrolyzed 34 % of benzamide, the liver extract 10.6 % in the phosphate solution and 6.8 % in the glycine solution.

The velocity of hydrolysis of BHA and BA cannot be compared on the basis of the above experiments since the concentrations of BHA and BA were different. In a later experiment series, in which liver extract was used for an enzyme preparation with 0.0174 mol BHA- and BA-solutions, it was noted that the hydrolysis of benzamide was considerably slower than that of benzohydroxamic acid (Fig. 1).

The liver extract used in the experiment was 2 weeks old (stored in an ice cupboard, with toluene) and it was much less active than the fresh liver extract used in the previous experiments.

Another similar experiment with a more active liver extract gave a similar result (Fig. 2).

The dependence of hydrolysis of BHA on pH was only roughly examined. At the highest pH used (7.4) the velocity with yeast autolysate was greatest, and at pH 6.0 abt. 2/3 of it. In acetate-buffer of pH 5.4 no hydrolysis occurred.

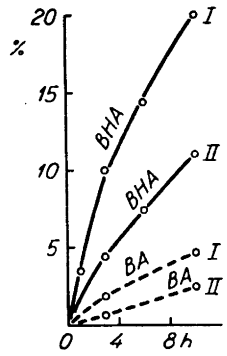

Fig. 1. Hydrolysis of benzohydroxamic acid (BHA, 0.0174M) and benzamide (BA, 0.0174M) with liver extract stored for 2 weeks in an ice-cupboard.

I in phosphate buffer (pH 7.3)
II in NaOH-glycine buffer (pH 7.3)

Borate buffer inactivated the enzyme concerned as appears from Table 3.

Table 3.

I 25 ml of phosphate solution (pH 7.3) with benzohydroxamic acid (0.002 mol) + 5 ml of yeast autolysate
II 25 ml of borate solution (pH 7.3) with benzohydroxamic acid (0.002 mol) + 5 ml of yeast autolysate

Time h	E-values I	II
0	142	144
12	52	144

Dialysis inactivates the yeast autolysate in a very high degree or practically completely. An $MgSO_4$-addition activated considerably the dialyzed autolysate in one experiment, in others again no activation was observed. The liquid outside the dialysis bag had no activating effect when evaporated to a small volume.

The dialysis of the liver extract did not cause any appreciable inactivation when BHA served as a substrate (Fig. 3).

It was proved by the following experiment that benzohydroxamic acid was really hydrolyzed to benzoic acid and hydroxylamine in the above experiments. The system: liver extract + benzohydroxamic acid (0.0174 M, pH 7.3) was examined qualitatively after 20 h in respect to benzoic acid and hydroxylamine. A drop of the solution formed a dark, non-fluorescent spot on a paper treated with 0.1 % fluorescein solution, thus benzoic acid had been formed. Determination of hydroxylamine showed that hydroxylamine, too, had been formed.

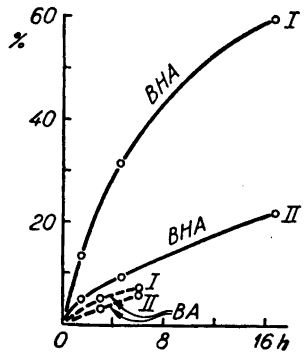

Fig. 2. As in Fig. 1, but with fresh liver extract.

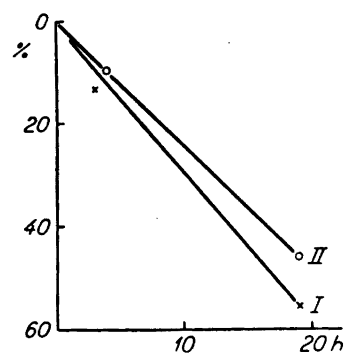

Fig. 3. Hydrolysis of BHA with undialyzed (I) and dialyzed (II) liver extract.

Synthesis

The first directive experiments were made with yeast autolysate (Experiment 8).

Experiment 8

I 122 mg of benzoic acid + 33 mg of NH_2OH in 25 ml of phosphate solution with pH 7.3 + 5 ml of yeast autolysate
II 366 mg of benzoic acid + 99 mg of NH_2OH in 25 ml of phosphate solution with pH 7.3 + 5 ml of yeast autolysate
III 430 mg of benzoic acid + 116 mgNH_2OH in 25 ml of phosphate solution with pH 7.3 + 5 ml of yeast autolysate
1 ml of reaction solution was used for each determination.

E-values from the BHA-determinations

Time h	I	II	III
0	3	0	8
2 ½	14	13	35
4 ½	20	11	37
6	29	15	37

The results suggest that the raw yeast autolysate synthesizes benzohydroxamic acid from benzoic acid and hydroxylamine.

In later experiments liver extract was used for an enzyme preparation (Experiment 13).

Experiment 13

0.5 ml of liver extract
0.1 ml of ATP-solution (0.1 μ M = 5.07 mg/10 ml)
0.1 ml of glutamine (10 μ M = 146.2 mg/10 ml)
0.1 ml of arsenate (10 μ M = 424.1 mg/10 ml)
0.5 ml of 0.1 M malic acid buffer (pH 6.4)
0.1 ml of manganous sulphate solution (0.1 μ M = 1.51 mg/10 ml)
0.1 ml of hydroxylamine solution (10 μ M)

were pipetted in a test tube. The mixture was kept at 35° C for 30 min, after which 1 m l of 10 % trichloracetic acid and 0.5 ml of 5 % ferric chloride were added. The protein precipitate was filtered. Filter S 50 was used in the photometric determinations of hydroxamic acid. Results:

	E
1. The basal solution	85
2. The basal solution, no glutamine	0
3. The basal solution, with boiled liver extract	0
4. The basal solution, benzamide instead of glutamine	37
5. The basal solution, benzoic acid instead of glutamine	35

The results reveal that the reactions

$$\text{glutamine} + NH_2OH \rightleftharpoons \text{glutamohydroxamic acid} + NH_3 \quad (1)$$
$$\text{benzamide} + NH_2OH \rightleftharpoons \text{benzohydroxamic acid} + NH_3 \quad (2)$$
$$\text{benzoic acid} + NH_2OH \rightleftharpoons \text{benzohydroxamic acid} + H_2O \quad (3)$$

have taken place. When the liver extract was boiled, no reaction occurred.

Because the synthesis from benzamide or benzoic acid is weak — in experiment 13 the E-values 37 and 35 correspond to about 20 γ BHA or about 1.5 % of the maximum synthesis — the experiment 14 was carried out on a larger scale and the BHA formed was extracted with ether from the reaction mixture. In this way BHA was strongly concentrated.

Experiment 14.

5 ml of liver extract
1 ml of ATP-solution (0.1 μM)
1 ml of benzamide (10 μM)
1 ml of arsenate (10 μM)
5 ml of 0.1 mol malic acid buffer (pH 6.4)
1 ml of manganous sulphate solution (0.1 μM)
1 ml of hydroxylamine solution, neutr. (10 μM)

After 30 min at 35° C, 2 ml of 50 % trichloracetic acid were added, the reaction mixture was centrifuged, its pH was adjusted to 7.2 with NaOH, and it was extracted with ether in a percolator. After evaporation of ether the extract was diluted with water to 2.2 ml. To the clear solution was added 0.5 ml of 5 % solution of ferric chloride and 1 ml of 10 % trichloracetic acid which brought the pH of the solution to about 2. An intense

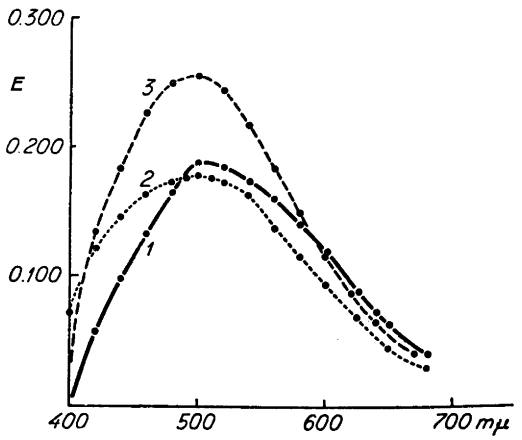

Fig. 4. Absorption spectrum of

1 synth. benzohydroxamic acid (m.p. 123° C)
2 ether extract in exp. 14 (benzamide + NH_2OH with liver extract)
3 basal solution in exp. 13 (glutamine + NH_2OH with liver extract).

red colour appeared and the absorption spectrum of the solution was determined between 400 and 700 mμ with a Beckman spectrophotometer. A parallel experiment was made with the same extract but without benzamide. The absorption of this solution did not show any maximum at 500 mμ, but rose sharply from 450 mμ to 400 mμ. It was subtracted from the absorption values given by the experiment proper.

On the basis of the absorption curve obtained (Fig. 4) the enzymatic synthesis of BHA is apparent.

In the following experiments the effect of ATP on the synthesis was examined. Since the undialyzed liver extract may contain ATP, the fact that the synthesis occurs without an addition of ATP does not prove that the reaction is independent of ATP. The results show, however, to what extent an addition of ATP may promote the synthesis and thus also the significance of ATP to the synthesis. The basal solution was the same as in experiment 13, likewise the experimental time and the method of analysis.

Experiment 15.

	E	Activation by ATP, %
1. The basal solution as in expt. 13	105	36
2. The basal solution, no ATP	77	
3. The basal solution, glutamic acid instead of glutamine	36	140

4. The basal solution, glutamic acid instead of glutamine, no ATP	15	
5. The basal solution, benzamide instead of glutamine	21	10
6. The basal solution, benzamide instead of glutamine, no ATP	19	
7. The basal solution, benzoic acid instead of glutamine	30	50
8. The basal solution, benzoic acid instead of glutamine, no ATP	20	

Experiment 16.

The basal solution was otherwise the same as in expt. 13 except that the liver extract was *dialyzed* for 24 h. The experimental arrangement was the same as in expt. 15.

	E	Activation by ATP, %
1. The basal solution	44	2
2. The basal solution, no ATP	43	
3. The basal solution, glutamic acid instead of glutamine	28	100
4. The basal solution, glutamic acid instead of glutamine, no ATP	14	
5. The basal solution, benzamide instead of glutamine	27	12
6. The basal solution, benzamide instead of glutamine, no ATP	24	
7. The basal solution, benzoic acid instead of glutamine	33	57
8. The basal solution, benzoic acid instead of glutamine, no ATP	21	

Fig. 5 illustrates the activation caused by ATP in experiment 16.

An addition of ATP has in all experiments greatly activated the synthesis of glutamohydroxamic acid from glutamic acid, hence, the indispensability of ATP for the synthesis is obvious, as the investigations of Speck and Elliott had revealed. The same addition had no activation on the synthesis of glutamohydroxamic acid from glutamine with dialyzed liver extract. This is in agreement with the results of Waelsch *et al.*

The activation of the synthesis of benzohydroxamic acid by the addition of ATP was distinct from benzoic acid, but slight from benzamide. It is therefore probable that the activation of the synthesis of hydroxamic acids through ATP is noticeably independent of the nature of the acids or amides,

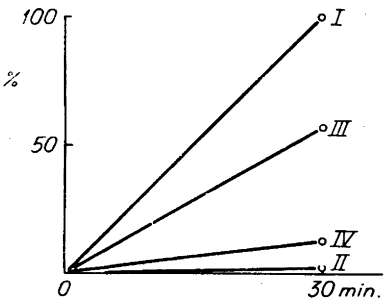

Fig. 5. Activation of the enzymatic synthesis of glutamo- and benzohydroxamic acids with dialyzed liver extract.

I Activation of the synthesis of glutamohydroxamic acid from glutamic acid.
II Activation of the synthesis of glutamohydroxamic acid from glutamine.
III Activation of the synthesis of benzohydroxamic acid from benzoic acid.
IV Activation of the synthesis of benzohydroxamic acid from benzamide.

which take part in the synthesis. The possibility that benzamide were hydrolyzed before the synthesis of benzohydroxamic acid must, however, be considered because ATP has shown a slightly activating effect when benzamide was used for the substrate.

SUMMARY

Hydrolysis of benzamide and benzohydroxamic acid was examined both with yeast autolysate and sheep-liver extract. Hydroxamic acid was hydrolyzed much more rapidly than amide. Benzoic acid and hydroxylamine were qualitatively identified as reaction products of the hydrolysis of benzohydroxamic acid.

No hydrolysis took place in borate buffer. In phosphate buffer the hydrolysis was more rapid than in glycine-NaOH buffer.

The synthesis of benzohydroxamic acid from hydroxylamine and benzoic acid or from benzamide was examined in detail with liver extract. The synthesis took place from both of them. ATP activated distinctly the synthesis from benzoic acid but only slightly that from benzamide.

The results presented in this paper show that the function of the enzymes, which catalyze the synthesis of hydroxamic acids, is not limited to glutamic and aspartic acids or their amides. The specificity of these enzymes is still obscure.

REFERENCES

1. Virtanen, A. I., and Alfthan, M. Unpublished.
2. Jacobsohn, K. P., and Soares, M. *Compt. rend. soc. biol.* **124** (1937) 1026.
3. Speck, J. F. *J. Biol. Chem.* **168** (1947) 403; **179** (1949) 1405.
4. Elliott, W. H. *Nature* **161** (1948) 128.
5. Waelsch, H., Borek, E., and Grossowicz, N. *Abstracts, Am. Chem. Soc.* 116th meeting 54 C (1949).

6. Waelsch, H., Owades, P., Borek, E., Grossowicz, N., and Schou, M. *Arch. Biochem.* **27** (1950) 237.
7. Grossowicz, N., Wainfan, E., Borek, E., and Waelsch, H. *J. Biol. Chem.* **187** (1950).
8. Stumpf, P. K., and Loomis, W. D. *Arch. Biochem.* **25** (1950) 451.
9. Stumpf, P. K., Loomis, W. D., and Michelson, C. *Arch. Biochem.* **30** (1951) 126.
10. Steward, T. C., and Street, H. E. *Plant Physiol.* **21** (1946) 155.
11. Roine, P. *Suomen Kemistilehti* **B 19** (1946) 113.
12. Fischer, E. *Anleitung zur Darstellung organischer Präparate*, 9th Ed. Braunschweig (1920) p. 39,
13. *Organic Synthesis* New York **II** (1947) p. 67.
14. Grassmann, W., and Mayr, O. *Z. physiol. Chem.* **214** (1933) 185.
15. Bray, H. G., James, S. P., Ryman, B. E., and Thorpe, W. V. *Biochem. J.* **42** (1948) 274.
16. Lipmann, F., and Tuttle, L. C. *J. Biol. Chem.* **159** (1945) 21.
17. Czáky, T. Z. *Acta Chem. Scand.* **2** (1948) 450.
18. Berenblum, I., and Chain, E. *Biochem. J.* **32** (1938) 295.
19. Gonnermann, M. *Pflügers Arch. ges. Physiol.* **89** (1902) 493; **95** (1903) 278.
20. Geddes, W. F., and Hunter, A. *J. Biol. Chem.* **77** (1928) 197.

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