[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY, NO. 273.]

ON THE PRODUCTS OF THE ACTION OF CERTAIN AMYLASES UPON SOLUBLE STARCH, WITH SPECIAL REFERENCE TO THE FORMATION OF GLUCOSE.

BY H. C. SHERMAN AND P. W. PUNNETT.

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Probably the first recognized case of what we now know as enzyme action was the observation by Kirchhoff (1814), confirmed and amplified by Dubrunfaut (1823) and by Payen and Persoz (1833), that a substance contained in barley malt is active in hydrolyzing starch with the production of reducing sugar. The sugar thus formed was for some time assumed to be glucose but later was shown by O'Sullivan[1] to be, at least chiefly, maltose. During the forty years since the publication of O'Sullivan's work several investigators have studied the kinds and amounts of products formed by the action of this and other amylases, but with widely varying results. The evidence as to the formation of glucose as a digestion-product of malt extract or pancreatic juice, for example, is conflicting[2] and among those who accept the affirmative evidence there is no apparent concensus of opinion as to whether the formation of glucose is due to the action of amylase or of a maltase simultaneously present. The general tendency to regard enzyme action as very highly specific has naturally favored the latter assumption and there has been no adequate evidence of the formation of glucose directly by amylase because in the work previously published on this subject the amylolytic agents used have been natural secretions, extracts, or simple alcohol precipitates which might have contained other enzymes along with the amylase. A reinvestigation of the question of the formation of glucose by the action of amylase was therefore undertaken.

Methods and Results.

The general plan of the present work was to allow pancreatic and malt amylases and the amylase of *Aspergillus oryzae*—chosen as representative

[1] *J. Chem. Soc.*, **25**, 579 (1872); **29**, 478 (1876); **30**, 125 (1876).

[2] V. Mering and Musculus, *Z. physiol. Chem.*, 1, 395 (1878); **2**, 403 (1878); Brown and Heron, *J. Chem. Soc.*, **35**, 596 (1879); Atkinson, *Chem. News*, **41**, 169 (1880); V. Mering, *Z. physiol. Chem.*, **5**, 185 (1881); Brown and Morris, *J. Chem. Soc.*, **47**, 527 (1885); Kellner, Mori and Nagaoka, *Z. physiol. Chem.*, 14, 296 (1889); Lea, *J. Physiol.*, 11, 226 (1890); Külz and Vogel, *Z. Biol.*, 31, 108 (1894); Brown and Morris, *J. Chem. Soc.*, **67**, 309, 709 (1895); Ling and Baker, *Ibid.*, **67**, 702, 739; **71**, 508; Krober, *Z. ges. Brauw.*, **18**, 325, 334 (1895); *Chem. Zentrbl.*, **66**, 1021; Chlodounsky and Sulc, *Jahr. Thierchem.*, **26**, 27 (1896); Brown, Morris and Millar, *J. Chem. Soc.*, **71**, 72, 109, 115 (1897); Brown and Millar, *Ibid.*, **75**, 315 (1899); Baker, *Ibid.*, **81**, 1177 (1902); Ling and Davis, *Ibid.*, **82**, 732 (1902); Clemm, *Pflüger's Archiv. f. d. ges. physiol.*, **89**, 517 (1902); Davis and Ling, *J. Chem. Soc.*, **85**, 16 (1904); Bierry, *Compt. rend.*, **141**, 146 (1905); **143**, 300 (1906); **146**, 417 (1907); Kita, *J. Ind. Eng. Chem.*, **5**, 222 (1913).

of the starch-splitting enzymes of the higher animals, higher plants, and fungi, respectively—to act upon soluble starch under comparable and carefully controlled conditions, and then to examine the digestion products for glucose both by means of the osazone reaction and by quantitative measurements of reducing and rotatory powers.

The enzymes have been used both in their commercial forms and after laboratory purification and, in general, have been allowed to act in the presence of such amounts of salt and phosphate as had been found in previous tests in this laboratory to be favorable to the action of each of these amylases. The ratios of enzyme to substrate usually approximated those with which we have to deal in determinations of diastatic power or in such studies of these enzyme hydrolyses as have been described in previous papers.[1] In all cases the temperature at which the enzyme was allowed to act was 40°.

The experiments here described were carried out in two series—the first in 1912, the second in 1914–15.

Series of 1912.—In the experiments of this first series, carried out with the coöperation of Mr. David F. Renshaw, attention was chiefly devoted to the evidence to be obtained by application of the osazone reaction. The previous work in this laboratory regarding the influence of concentration of glucose and presence of maltose[2] was supplemented by the following observations: Using 0.4 g. of freshly purified phenylhydrazine hydrochloride with 0.6 g. of sodium acetate in a volume of 4 cc. it was found possible to obtain recognizable osazone from 10 mg. of glucose, after one hour's heating in the boiling water bath; but in the presence of several times its weight of maltose the glucose reacted much less readily.

When the amount of sodium acetate was increased to 0.8 g. (for 4 cc. of the test solution) the test appeared to be improved; this amount was therefore used in all subsequent experiments.

On applying the test to mixtures of glucose and maltose totaling 0.20 g. (the "standard" weight of sugar for the application of the osazone test in a volume of 4 cc.) the following results were obtained:

Percentage composition of sugar mixture, Glucose.....	10	9	8	7	6	5	4
Maltose.....	90	91	92	93	94	95	96
Minutes of heating for appearance of glucosazone.....	17	22	26	30	33	51	60

These results indicate the possibility of detecting by this application of the osazone test as little as 4% of glucose in a mixture of glucose and maltose alone, and of roughly judging the amount of glucose when present in somewhat larger proportion in such a mixture.

Dextrin was found to cause a marked retardation, even when present

[1] THIS JOURNAL, 32, 1073, 1087; 33, 1195; 34, 1104; 35, 1617, 1784, 1790; 37, 623, 643, 1305.

[2] Sherman and Williams, *Ibid.*, 28, 629 (1906).

to the extent of only 10% of the total carbohydrate, and if in excess of that proportion, it rendered the formation of the glucosazone very uncertain. Since it was to be expected that the mixtures of digestion products obtained by amylase action at 40° would contain at least 10% of dextrin, and probably 20% even when the action was long continued, it appeared impossible to use the digestion mixtures directly for the osazone tests. Two methods for removing the dextrin were suggested by work of previous investigators—to precipitate the dextrin by pouring the solution into alcohol; or to evaporate the solution to dryness and extract with alcohol. The precipitation method did not give good results when tried on known mixtures of glucose, maltose and dextrin—possibly because of adsorption of glucose by the precipitated dextrin or because of partial solubility of the dextrins in the alcohol solution. The second method proved better; practically no dextrin was extracted by the alcohol, and the relative solubility of glucose in hot 95% alcohol (about five times that of maltose according to Lippman), made possible the extraction of most of the glucose by the use of small amounts of the solvent. The procedure, as finally worked out, was as follows:

The digestion mixture, which had been kept at 40° for the required time, was heated to boiling to stop the enzyme action and (usually after the determination of the rotatory and reducing powers) was evaporated to dryness on a water bath with the addition of some clean sand to render the mass more porous and facilitate its subsequent extraction. The dry mass of carbohydrates, salts and sand was then boiled for two minutes, with constant and vigorous stirring with 20 cc. of 95% ethyl alcohol. The alcohol extract was decanted off while hot and evaporated to dryness on the water bath. The sugar thus extracted was weighed and dissolved in twenty times its weight of water and 4 cc. of this solution used for each osazone test. If the digestion has been long continued, about 10–15% of the mass is extracted in this way. If the starch conversion is very incomplete, it is generally necessary to repeat the extraction process in order to obtain a sufficient quantity of reducing sugar for satisfactory testing.

This method was applied to the digestion products resulting from the action of the following amylase preparations: (1) a high grade commercial pancreatin powder furnished by Parke, Davis and Company; (2) a highly purified and very active preparation of pancreatic amylase made in this laboratory; (3) malt amylase preparations purified in the laboratory but not representing as high a degree of activity as was reached later; (4) a preparation, kindly furnished us by Dr. J. Takamine, which contained the amylase of *Aspergillus oryzae* and showed about four times the diastatic power of ordinary commercial takadiastase. *In all of these cases* the sugar extracted from the digestion mixture gave an osazone reaction for glucose when tested as above described. In nearly all cases, however, the reaction was very slight. Only in the extract from the products of long-continued (18 hours) action of takadiastase (containing the amylase of *Aspergillus oryzae*) was there indication of one-tenth as much glucose as maltose.

Several different samples of commercial pancreatin were allowed to act upon soluble starch for 39 hours at 40° and the digestion products then tested as above described. In all of these cases the osazone reaction gave evidence of glucose.

Series of 1914–5.—In this second series of experiments the enzymes were used in such concentrations as to be as nearly as practicable equivalent in amyloclastic power, the quantity of enzyme preparation employed in each case being such as would digest the starch present to disappearance of blue or violet color-reaction in 30 minutes. Activating salts were used as in the first series, and the general arrangement of the experiments was the same as before, the digestion products being evaporated on sand and extracted with alcohol. Special attention was devoted to the purification of the water and salts used. Digestions were conducted at 40° and usually for 24 hours—48 times as long as was required for the digestion of the starch to products giving no blue or violet color with iodine. The reducing and rotatory powers of the organic solids of the digestion mixture and of its alcohol extract were determined, and compared with those of pure maltose. A higher reducing power or lower rotatory power than would be shown by an equal weight of pure maltose was taken as evidence of the presence of glucose in the solids extracted by the 95% alcohol from the evaporated digestion mixture (for if any dextrin had been extracted the effect would have been to alter each of these properties in the opposite direction).

The procedure for each experiment was as follows: A quantity of soluble starch, usually equivalent to 8 g. of dry matter, was dissolved in pure boiling water and, after the addition of the measured amount of the salts necessary to give the proper concentration of activator in the final solution, the solution made up to such a volume as, after the subsequent addition of the required amount of enzyme solution, would give a starch concentration of 2% in the digestion mixture. The flask was then immersed in the constant temperature bath until it had reached 40°, after which the enzyme solution was added, the flask stoppered with a plug of cotton and left in the bath for 24 hours.

At the expiration of this time it was removed, boiled to stop the enzyme action and filtered to remove the small amount of suspended matter. Considerable difficulty was experienced in this filtration. The best method was found to be by the use of Gooch crucibles containing thin pads of washed asbestos. By using three such crucibles in rotation it was possible to filter 400 cc. of solution in a few hours. This insoluble suspended matter always amounted to about 1% of the weight of the starch.

The filtered solution was often greater than the required volume and the subsequent evaporation usually produced a gelatinous precipitate, of a few milligrams weight, which was also filtered out.

The filtered solution was then made up to volume and an aliquot portion (one-tenth) was withdrawn for use in the determination of rotatory and reducing powers. Another such portion was evaporated to dryness on a water bath in a weighed glass evaporating dish containing some ignited, clean sand and then dried to constant weight by heating at 70° under a reduced pressure of 40 to 100 mm. This weight of

material corrected for the weight of enzyme and of dry activator gave the total soluble carbohydrates.

The remaining four-fifths of the solution was also evaporated on the water bath, with the addition of sand. This mixture of carbohydrate, activating salt and sand was next extracted on the water bath with boiling 95% alcohol in 20 cc. portions. The alcohol extract was evaporated to dryness, dissolved in water, and made up to a volume of 100 cc. with filtration if necessary. Ten cc. of this solution were withdrawn and used for the determination of solids as in the case of the original solution. The rotatory and reducing powers were determined as described above.

The residue left with the sand after the alcohol extraction was dissolved in hot water, filtered, and made up to 250 cc. Fifty cc. (or sometimes 25 cc.) were used for the total-solids determination and the rotatory and reducing power determined on a portion and calculated to the basis of dry carbohydrate material.

Finally the remainder of the extract solution was evaporated until each cubic centimeter contained 0.1 g. of carbohydrate and 2 cc. of this solution used in each osazone test—using 0.3 cc. of pure phenylhydrazine, 0.2 cc. of 87% acetic acid (equivalent to the phenylhydrazine base), 0.3 g. of sodium acetate, 2.0 cc. of sugar solution and 1.5 cc. of water, giving a total volume of 4 cc.

Summary of Results.

Pancreatic amylase tested both in the form of commercial pancreatin and as a highly active purified preparation made by Miss M. D. Schlesinger from this same pancreatin as previously described[1] resulted in an extract having greater reducing power than pure maltose which, as explained above, is taken as evidence of the presence of glucose. The osazone reaction for glucose was negative in all of these extracts, indicating that the yield of glucose was small as compared with the large amount of maltose produced. The close agreement of results obtained from the action of the commercial pancreatin and of the purified amylase made from it suggests that such glucose production as occurs is attributable to the action of the amylase rather than of a separate enzyme (maltase); for it is hardly reasonable to suppose that a maltase, present as a separate substance in the pancreatin, would have remained with the amylase in unchanged ratio throughout the purification process.

Malt amylase was tested in the form of a purified preparation[2] and parallel experiments were made with malt extract. The measurements of reducing and rotatory powers of the sugars extracted as above described indicated that, along with a large amount of maltose, a small proportion of glucose had been formed both by the malt extract and by the purified malt amylase. The osazone tests for glucose were negative in these cases, the amount of glucose in the digestion mixture being too small to be demonstrated by this test, even as applied to the solids obtained by alcohol extraction.

[1] Preparation 62 II made as described by Sherman and Schlesinger (THIS JOURNAL, 34, 1105 (1912)) and showing a diastatic power of 3313, equivalent to about 5000 on Lintner's scale.

[2] Preparation 111A. Sherman and Schlesinger, THIS JOURNAL, 37, 648 (1915).

It thus appears that both pancreatic and malt amylases, even when highly purified, are not limited to the formation of maltose but may form some glucose as well; but that under conditions such as obtained in the determination of diastatic power the proportion of glucose formed is so minute as to be practically negligible in comparison with the much greater amounts of maltose formed.

Takadiastase and the partially purified **amylase of Aspergillus oryzae** prepared from it by Dr. A. P. Tanberg,[1] when tested in a manner parallel to the experiments with pancreatic and malt amylases just described, produced a larger yield of glucose, but here also maltose was the chief digestion product even though the digestion was continued 40 to 50 times as long as was required for the disappearance of the starch-iodine reaction.

When commercial takadiastase, in quantity sufficient to digest all the starch present in one-half hour to disappearance of the starch-iodine reaction, was allowed to act for 24 hours (at 40° and in the presence of activating salts), the analysis of the resulting digestion mixture and its alcoholic extract indicated that of the total carbohydrate present more than half was maltose, about one-fourth was glucose, and somewhat less than one-fourth was dextrin.

The amylase preparation made from the takadiastase and representing a considerable degree of purification and concentration of both amyloclastic and saccharogenic power, formed in a parallel experiment a somewhat smaller proportion of glucose. This may be due either to the elimination of a maltase in the purification process or to the fact that the purified amylase is less stable than the commercial and therefore would not show as great relative activity in a long as in a short digestion experiment.

With all three types of amylase it is noticeable that considerable quantities of dextrin still appear among the digestion products, even when the digestion is allowed to proceed forty to fifty times as long as is required for the disappearance of the starch-iodine reaction.

On the other hand, many of our experiments have shown a larger yield of reducing sugar and smaller yield of dextrin than would correspond with the "resting stage" described by some of the earlier investigators.

The different enzymes all left about 1% of the weight of the "soluble" starch as a very finely divided difficultly filtrable insoluble residue.

There are indications that dextrin, maltose and glucose may not constitute the sole products of the action of amylases upon soluble starch.

Conclusion.

The experiments lead to the conclusion (1) that any of the amylases

[1] The material here used was Dr. Tanberg's Preparation No. 15 having a diastatic power of 194 ("new scale") which is about 8 times the activity of commercial takadiastase but somewhat less than half the maximum activity thus far attained. See Sherman and Tanberg, THIS JOURNAL, 38, 1638.

		Experiments with Commercial Takadiastase.			Experiments with Malt extract.			
		No. 3.	No. 4.	No. 5.	No. 6.	No. 7.	No. 8.	
1.	Dry starch (g.)	8.0	8.0	8.0	8.0	8.0	8.0	
2.	Insoluble (g.)	0.1157	0.1103	0.0994	0.1140	0.1039	0.0904	
3.	Insoluble (%)	1.44	1.38	1.24	1.43	1.30	1.13	
4.	Sol. carboh. in T (g.)	8.488	8.393	8.313	8.193	8.154	8.204	
5.	Total carb. in T (g.)	8.6037	8.5033	8.4124	8.307	8.258	8.2944	
6.	Increase (g.)	0.6037	0.503	0.4124	0.307	0.258	0.2944	
7.	Increase (%)	7.55	6.28	5.15	3.84	3.22	3.68	
8.	$[\alpha]_D$ of T	126.49°	126.7°	129.1°	142.9°	141.95°	142.7°	
9.	"R" in maltose (g.)	8.245	8.105	8.290	7.610	7.670	7.920	
10.	"R" in glucose (g.)	5.040	4.950	5.060	4.645	4.680	4.830	
							Part I.	Part II.
11.	Alc. used in extraction (cc.)	200	300	300	200	150	200(abs.)	100(95%)
12.	Carboh. in X (g.)	2.907	3.864	4.310	3.382	3.666	0.566	1.633
13.	Corresp. carboh. in T (g.)	3.6338	4.830	5.388	4.2275	4.583	0.707	2.041
14.	$[\alpha]_D$ of X	102.12°	108.6°	107.02°	128.2°	132.5°	130.7°	130.1°
15.	"R" in maltose (g.)	3.272	4.490	4.901	3.360	3.717	0.568	1.685
16.	"R" in glucose (g.)	1.995	2.739	2.990	2.048	2.270	0.345	1.029
17.	Carboh. in R (g.)	3.6594	2.701	2.153	3.306	2.844	2.712	1.640
18.	Corresp. wt. in T (g.)	4.574	3.376	2.691	4.132	3.555	3.390	2.05
19.	$[\alpha]_D$ of R	152.8°	159.7°	183.7°	150.8°	153.8°	142.6°	151.2°
20.	"R" of R in maltose (g.)	3.256	2.154	1.692	2.686	2.470	2.582	1.480
21.	"R" in glucose (g.)	1.988	1.314	1.032	1.652	1.506	1.576	0.904
22.	Corresp. X and R (g.)	8.208	8.206	8.079	8.360	8.138	8.188	
	Sol. carboh. in T + malt ext. solids	....	....	....	8.345	8.315	8.364	
23.	Loss carboh. (g.)	0.280	0.187	0.234	(Gain 0.015)	0.177	0.176	
	Osazone tests in hot soln. (heated 2 hrs.)	....	....	....	Negative	Negative	Negative	

	Purified Malt Amylase. (Preparation 111A.)		Amylase of Aspergillus oryzae.		Commercial Pancreatin. (Pancreatin 6.)		Purified Pancreatic Amylase (62II).	
	No. 9.	No. 10.	No. 11.	No. 12.	No. 13.	No. 14.	No. 15.	No. 17.
Enzyme (mg.)	4.6	7.7	3.3	3.75	1.71	6.0	0.2	0.6
Dry starch (g.)	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Insoluble (g.)	0.106	0.079	0.030	0.0864	0.0689	0.1014	0.0891	0.0966
Insoluble (%)	1.33	0.99	0.38	1.08	0.86	1.27	1.11	1.21
Sol. carbohy. in T (g.)	8.234	8.276	8.235	8.222	8.167	8.264	8.116	8.211
Total carbohydrate (g.)	8.340	8.355	8.265	8.308	8.236	8.365	8.205	8.308
Increase (g.)	0.340	0.355	0.265	0.308	0.236	0.365	0.205	0.308
Increase (%)	4.25	4.44	3.31	3.85	2.95	4.56	2.56	3.85
$[\alpha]_D$ of T	147.95°	146.9°	148.2°	144.8°	156.6°	152.9°	155.2°	157.5°
"R" in maltose (g.)	7.210	7.140	7.220	7.465	6.340	6.515	6.030	5.955
"R" in glucose (g.)	4.400	4.360	4.410	4.495	3.870	3.975	3.680	3.635
Alc. for extraction—95% (cc.)	100	100	100	100	100	100	100	100
Carbohydrate in X (g.)	2.119	1.884	2.542	1.850	1.845	1.602	1.206	1.303
Corresp. in T (g.)	2.659	2.355	3.175	2.313	2.306	2.005	1.508	1.629
$[\alpha]_D$ of X	130.3°	125.9°	136.4°	125.1°	138.2°	139.6°	137.0°	141.1°
"R" in maltose of X (g.)	2.216	1.922	2.736	2.044	2.070	1.638	1.248	1.304
"R" in glucose of X (g.)	1.352	1.172	1.668	1.246	1.262	1.000	0.762	0.794
Carbohydrate in R (g.)	4.540	4.694	3.983	4.748	4.597	4.647	5.164	5.234
Corresp. in T (g.)	5.674	5.868	4.978	5.935	5.746	5.809	6.455	6.543
$[\alpha]_D$ of R	151.8°	154.0°	156.0°	151.2°	161.3°	164.9°	160.8°	162.75°
"R" in maltose of R (g.)	3.390	3.793	3.073	3.953	3.058	3.568	3.748	3.465
"R" in glucose of R (g.)	2.068	2.303	1.873	2.413	1.865	2.178	2.288	2.115
Corresp. X and R (g.)	8.333	8.223	8.153	8.248	8.052	7.814	7.963	8.172
Loss carboh. in process (g.)	0.99 (gain)	0.053	0.082	0.026(gain)	0.184	0.450	0.153	0.039
Osazone tests	Negative in 2 hrs.	Negative in 2 hrs.	17 min.	12 min.	Negative	Negative	Negative	Negative
Barfoed tests	....	....	....	Positive	White ppt. no red	Same as No. 13	Same as No. 13	Same as No. 13

here studied may form some glucose, (2) that in such conditions as obtain in the usual determinations of diastatic power the yield of maltose so far predominates as to justify the custom of calculating the reducing powers of the digestion products as if due to maltose alone.

Detailed Data of the Principal Experiments of the Series of 1914–5.

In the above tables, the data are recorded under abbreviated captions, the significance of which are as follows:

(1) is the weight of dry starch used. (2) is the weight of insoluble matter filtered out. (3) is the percentage of the dry starch consisting of such insoluble matter. (4) is the weight of soluble carbohydrates in the total solution; *i. e.*, total solids less activation salts, less enzyme. (5) is the sum of (2) and (4). (6) is the difference between (5) and (1). (7) is the percentage increase in weight over the weight of dry starch used. (8) is the specific rotatory power for the soluble carbohydrates in the total solution ("T"). (9) is the reducing power expressed in terms of maltose, and (10) in terms of glucose for the whole solution. (11) is the amount of alcohol used for the extraction. (12) is the weight of carbohydrates in the extract solution ("X") and (13) is the weight equivalent to the total solution; *i. e.*, (12) $\times$ 1.25, since the extraction was made on four-fifths of the original material. (14) is the specific rotatory power for the carbohydrates in the extract solution. (15) and (16) are the reducing power of the extract solution expressed in terms of maltose and glucose, respectively. (17) is the weight of carbohydrate in the residue solution ("R"), *i. e.*, total solids less four-fifths the weight of added activator. (18) is the portion of soluble carbohydrates in the original total solution corresponding to the weight in the residue solution. (19) is the specific rotatory power of the carbohydrates in the residue solution and (20) and (21) their reducing power expressed in terms of maltose and glucose, respectively. (22) is the sum of (13) and (18) and when subtracted from (4) gives the loss of material (23) due to the process.

LABORATORY OF FOOD CHEMISTRY.

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EXPERIMENTS UPON STARCH AS SUBSTRATE FOR ENZYME ACTION.

BY H. C. SHERMAN AND J. C. BAKER.

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The forms in which starch has been used as substrate for enzyme action may be grouped as: (1) Natural starch dispersed in water under different conditions of time, temperature, and pressure but without "chemical" treatment; (2) starch which has been subjected to the action of acid (Lintner)[1] or to other chemical treatment (Wolff and Fernbach)[2] to render it "soluble;" (3) fractions of the starch substance which have been separated from the remainder by sedimentation (Tanret),[3] or pre-

[1] *J. prakt. Chem.*, [2] 34, 378 (1886); see also Ford, *J. Soc. Chem. Ind.*, 23, 414 (1904).

[2] *Compt. rend.*, 140, 1403 (1905); 143, 363, 380 (1906).

[3] *Bull. soc. chim.*, [4] 17, 83 (1915).