trifluoride were made as soon after filling the bulbs as possible since it slowly attacks glass with the formation of silicon tetrafluoride. The other substances used were of the best grades obtainable.

A number of determinations were made on both reactions and five values for each were finally selected as being the best. The average of these for reaction (1) is $\Delta H_1 = -64{,}500$ cal. with a mean error of 760 cal., and for reaction (2) $\Delta H_2 = -10{,}700$ cal. with a mean error of 800 cal.

The following heats of formation[1] were used in the calculations. NaOH(1 *N*), 112,210 cal.; H_2O(l), 68,387 cal.; As_2O_3(s), 156,000 cal.;[2] NaF(s), 137,600 cal. When these thermal data are combined with those determined for reactions (1) and (2), the following thermochemical equation may be written

$$As(s) + 3/2F_2(g) = AsF_3(l) \qquad (3)$$
$$\Delta H = -198{,}300 \text{ cal.}$$

Although no experimentally determined value for the heat of vaporization of arsenic trifluoride (b. p. 63°) is available, a fairly reliable value of −7000 cal. results if Trouton's constant is taken as 21 cal./deg. This, combined with equation (3), yields 191,300 cal. as the heat of formation of AsF_3(g).

It is now possible to calculate a provisional value for the standard free energy of formation of gaseous arsenic trifluoride at 25°. The standard virtual entropies of As(s),[2] F_2(g)[3] and AsF_3(g)[4] at 25° are 8.4 cal./deg., 48.0 cal./deg. and 69.2 cal./deg., respectively. The free energy equation is then

$$As(s) + 3/2F_2(g) = AsF_3(g) \qquad (4)$$
$$\Delta H = -191{,}300 \text{ cal.} \quad \Delta F^\circ_{298} = -188{,}000 \text{ cal.}$$

Rough measurements on the vapor pressure of AsF_3(l) showed it to be about 150 mm. at 23.5° and therefore the free energy of vaporization is about 1000 cal. When combined with equation (4) there results the rough value −189,000 cal. for the free energy of formation of AsF_3(l). Inasmuch as arsenic trifluoride is of considerable importance in reactions used for the preparation of other fluorides, these free energy values may find application in determining beforehand whether a proposed reaction is possible.

(1) "International Critical Tables," Vol. V, pp. 161, 177, 180, 200.
(2) C. T. Anderson, THIS JOURNAL, **52**, 2296 (1930).
(3) Yost and Hatcher, *J. Chem. Educ.*, **10**, 350 (1933).
(4) Yost and Anderson, *J. Chem. Phys.*, **2**, 624 (1934); Yost and Sherborne, *ibid.*, **2**, 125 (1934).

PUBLICATION NO. 450
GATES CHEMICAL LAB.
CALIFORNIA INST. OF TECH.
PASADENA, CALIFORNIA RECEIVED FEBRUARY 23, 1935

[CONTRIBUTION FROM THE FLEISCHMANN LABORATORIES]

A General Method for Determining the Concentration of Enzyme Preparations[1]

BY W. R. JOHNSTON AND S. JOZSA

It is generally recognized that the "enzyme unit" method as introduced by Willstätter and Kuhn[2] is the most satisfactory way we have of measuring enzyme quantity.

In applying the method, however, a number of authors have defined their enzyme units in terms of the monomolecular "constants" for the reactions being studied. This application is not strictly correct since it has been established by numerous workers that in general the unimolecular law does not apply to enzyme reactions and the "constants" cannot be used as an accurate measure of enzyme quantity. This is true in the case of invertase, urease, alpha-amylase and many other enzymes.

Other investigators have defined units in terms of time values required for a definite percentage hydrolysis of the substrate by a given amount of enzyme. These units lead to sufficiently accurate measures of enzyme quantity but are not very satisfactory because of the relatively long time required for measurement and calculation.

In attempting to measure the concentration of α-amylase in various malt preparations we developed an enzyme unit method which is of general applicability and which permits rapid, accurate measurements of enzyme quantity. The method is based upon the fact that in the case of several enzymes the initial rate of action on a given substrate is directly proportional to the amount of enzyme acting. We have found this to be true in the case of α- and β-amylase and yeast invertase and other workers have demon-

(1) Presented before the Division of Biological Chemistry at the 86th meeting of the American Chemical Society, Chicago, Ill., September 10–15, 1933.
(2) Willstätter and Kuhn, *Ber.*, **56**, 509 (1923).

strated it with other enzymes. The development of the method in connection with α-amylase is presented in this paper.

Alpha-amylase, the liquefying component of the amylase system, has been studied extensively for many years. In most cases the activity of the enzyme has been expressed in terms of liquefying power. Liquefying power has always been a loosely defined term, signifying in general an average rate of liquefaction of a given starch substrate. It has never been emphasized that liquefying power can only be defined precisely when the instantaneous rate of liquefaction is taken into consideration. Kjeldahl and others have pointed out that by using a sufficiently concentrated substrate one can observe approximate proportionality between amount of starch converted and the time of reaction. However, the linear relation is not exact and in general the rate of liquefaction of starch paste varies continually as the reaction proceeds. Hence, the rate of doing work or the liquefying power varies throughout the reaction period and must be defined at a given time in order to be precise. The liquefying power is a function not only of the substrate, the enzyme concentration, etc., but also of the time of observation. The most logical time of observation is the beginning of the reaction or zero time. At this point all inhibiting factors are at a minimum, making zero time the ideal reference point. In order to make accurate measurements of liquefying activity which will serve for practical application we shall define the liquefying power of an α-amylase system as the calculated rate of liquefaction of starch by one gram of enzyme preparation at zero time under the specified conditions. This definition is precise and useful in an important respect.

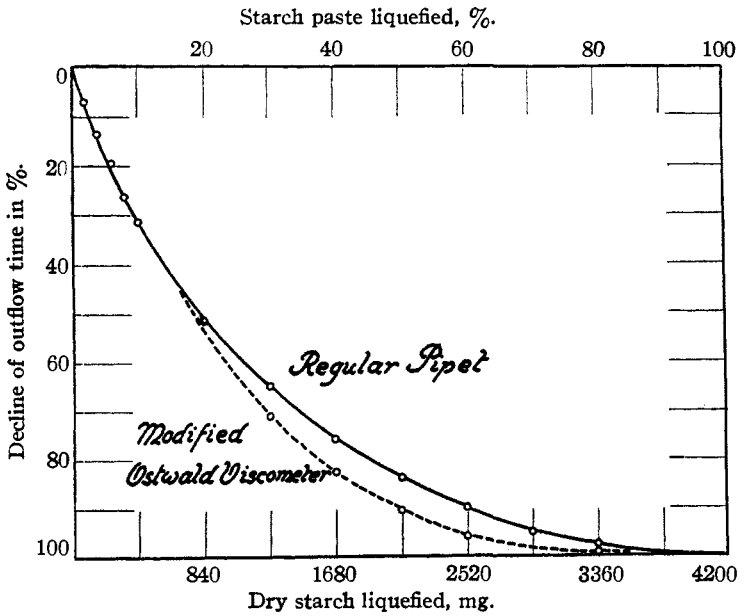

Fig. 1.—Standard liquefying curve.

We find experimentally that the rates of liquefaction at zero time are directly proportional to the enzyme concentration over a wide range. This gives us an excellent means of estimating enzyme concentrations. In order to measure enzyme concentrations we shall define an α-amylase unit termed a *liquefon* as that amount of starch-liquefying enzyme which will convert the standard starch paste at the rate of 25 mg. of dry starch per minute at zero time under the specified conditions. A determination of the number of liquefons per gram of preparation gives us an exact measure of the enzyme content and at the same time gives us a measure of the liquefying power at zero time. We believe that the liquefon is a rational and logical unit since our present knowledge permits us to characterize enzymes only by the type of reaction they catalyze and the rate at which the reaction proceeds.

The experimental evidence necessary for this formulation has been obtained by a careful study of initial reaction rates.

Experimental

The method of preparation of the starch paste used as a substrate has been fully described in another paper ("The Determination of α-Amylase," S. Jozsa and W. R. Johnston, to be published in *Analytical Edition of Industrial and Engineering Chemistry*). In order to estimate the amount of starch liquefied we have again used the same empirical method described in our paper on the determination of α-amylase. However, in order to make the rate measurements near the start of the reaction it was necessary to use a modified Ostwald viscometer which had a relatively short outflow time. The instrument differed from the usual viscometer in that the capillary was replaced by a spiral glass tube, approximately 4 mm. in diameter and 60 cm. long, which permitted rapid flow of the viscous paste and allowed measurements about once per minute, after the first two to four minutes of reaction time. A calibration curve was made in the usual manner by preparing a series of mixtures of fully liquefied paste and stirred starch paste. The calibration curve for standard pipets and for

the modified Ostwald viscometer are shown in Fig. 1. The curves differ somewhat, which is surely to be expected. The data plotted for the modified Ostwald viscometer curve are given in Table I.

It should be emphasized that the modified viscometer employing the spiral tube was carefully calibrated for use in the manner described. It was used only to obtain data on the initial rates of liquefaction of starch and is not to be considered as a standard instrument or one recommended for further use. Other instruments might well be used to establish the rate curves.

TABLE I

Starch liquefied, %	Dry starch liquefied, mg.	Decline in outflow time, %
2	84	7.2
4	168	13.8
6	252	20.1
8	336	24.8
10	420	30.3
20	840	52.7
30	1260	71.2
40	1680	82.6
50	2100	90.2
60	2520	95.5
80	3360	99.1
100	4200	100.0

The milligrams of starch liquefied were obtained from the calibration curve and the results plotted to give the rate curves. It was found that the rate at which the starch was liquefied at zero time was directly proportional to the concentration of enzyme.

In all the calculations the results are referred to 100 g. of paste and 10 cc. of infusion. The infusions varied in concentration from 0.75 mg. per 10 cc. to 7.00 mg. per 10 cc. They were prepared by diluting the proper weight of a concentrated malt infusion to one liter with distilled water and then further diluting 100 cc. of the first dilution to one liter. A standard concentrate was set aside for all the rate experiments and was carefully preserved at a low temperature to prevent any changes in enzyme content. Table II gives some experimental results. The points near the origin are the least accurate since the time errors are large. At very low enzyme concentrations the first point could be obtained accurately only after six or seven minutes. The total range signifies the drop in outflow time after complete liquefaction. In the viscometer used the blanks usually gave an outflow time of about seventy-nine or eighty seconds. The blanks were always prepared with boiled enzyme infusion cooled to 21°. We used boiled enzyme infusion in order to approximate as closely as possible the effect of the enzyme infusion on the viscosity of the paste. The boiled infusion always lowered the viscosity of the paste more than distilled water.

TABLE II

Enzyme concentrate, 7 mg./10 cc. Total range, 80.2 − 1.2 = 79.0 sec.								
Time, minutes	2.67	4.13	5.43	6.63	7.80	8.83	9.73	12.66
Starch liquefied, mg.	357	571	710	815	907	995	1054	1247
Enzyme concentrate, 5 mg./10 cc. Total range, 82.5 − 1.2 = 81.3 sec.								
Time, minutes		1.98	3.50	4.85	6.00	7.18	8.22	9.25
Starch liquefied, mg.		143	344	470	580	672	748	815
Enzyme concentrate, 2.5 mg./10 cc. Total range, 79.0 − 1.2 = 77.8 sec.								
Time, minutes	3.67	5.10	6.42	7.75	9.00	10.16	11.00	13.30
Starch liquefied, mg.	172	252	319	374	437	483	524	610
Enzyme concentrate, 1.25 mg./10 cc. Total range, 78.6 − 1.2 = 77.4 sec.								
Time, minutes	6.00	7.77	9.55	11.47	13.00	16.70	18.30	22.15
Starch liquefied, mg.	155	193	214	239	277	328	365	445
Enzyme concentrate, 0.75 mg./10 cc. Total range, 80.2 − 1.2 = 79.0 sec.								
Time, minutes	5.30	7.40	9.17	11.00	12.67	16.71	18.37	20.11
Starch liquefied, mg.	94	105	126	147	160	178	202	218

The viscometer was placed in a bath at 21.0 ± 0.1° and the rate experiments were made as follows. Fifteen cubic centimeters of enzyme infusion was stirred (using the high speed stirrer) into 150 g. of starch paste, the time of stirring being noted. The infusion was stirred in for a time corresponding to that used in the blank determinations as in the case of the pipet measurements described in the paper on the determination of α-amylase. The paste was always precooled so that after stirring, the mixture was always within 0.1 to 0.2° of 21.0°. After stirring 25 cc. of paste was pipetted into the viscometer and the outflow times measured as often as possible for the first ten to fifteen minutes. The time measurements were corrected for the interval the mixture remained in the upper bulb by subtracting half the outflow time from the time after the completion of a viscosity reading. Thus the actual rate at any given time was more closely approximated.

The outflow time of completely liquefied paste was consistently 1.2 seconds. The data for the concentration of 2.5 and 0.75 mg./10 cc. are plotted in Fig. 2.

All the data were plotted on a large scale and the initial slopes measured. The following table shows that within the experimental error of about 4–5% the initial rates are directly proportional to enzyme concentration.

TABLE III

Concn. infusion, mg./10 cc.	7.0	5.0	2.5	1.25	0.75
Liquefaction of starch at zero time in mg. per minute	142	104	50	25	15

It was observed that sodium chloride increased the rate of action of a given enzyme preparation. This effect was

TABLE IV

Enzyme concentrate, 7 mg./10 cc. Total range, 82.4 − 1.2 = 81.2 sec.										
Time, minutes	2.42	3.77	5.00	6.10	7.27	8.21	9.15	10.97	14.65	16.95
Starch liquefied, mg.	412	609	752	869	966	1046	1126	1264	1499	1571
Enzyme concentrate, 5 mg./10 cc. Total range, 78.8 − 1.2 = 77.6 sec.										
Time, minutes	2.20	3.60	4.95	6.10	7.12	8.25	9.40	11.00	12.25	15.03
Starch liquefied, mg.	206	388	533	630	718	806	874	995	1067	1222
Enzyme concentrate, 2.5 mg./10 cc. Total range, 78.9 − 1.2 = 77.7 sec.										
Time, minutes		3.90	5.48	6.90	7.21	8.36	8.60	9.58	9.95	10.88
Starch liquefied, mg.		168	256	344	382	416	445	483	508	546
Enzyme concentrate, 1.25 mg./10 cc. Total range, 80.3 − 1.2 = 79.1 sec.										
Time, minutes			4.05	6.00	8.80	10.60	12.25	14.75	17.80	21.27
Starch liquefied, mg.			105	147	223	262	298	361	403	504
Enzyme concentrate, 0.75 mg./10 cc. Total range, 80.4 − 1.2 = 79.2 sec.										
Time, minutes				4.27	8.05	9.87	11.70	13.47	15.73	21.00
Starch liquefied, mg.				67	139	164	193	223	254	323

studied previously by Jozsa and Gore.[3] Several concentrations of sodium chloride were tried in order to ascertain the optimum concentration for maximum activation. A concentration of 25 g. of sodium chloride per liter in the first dilution of a sirup was found to be adequate for complete activation. The salt seems to prevent adsorption of the alpha amylase and tends to stabilize infusions. More accurate results may be obtained by the use of sodium chloride so the rate experiments were repeated using the optimum sodium chloride concentration of 25 g. per liter. The final dilution always had a concentration of 2.5 g. of sodium chloride per liter. The data are given in Table IV. The initial rate is increased about 10% over that of a distilled water infusion of the same concentration, and the rate is maintained over a longer period of time in the presence of sodium chloride. This is shown clearly in Fig. 2. The bending of the rate curves in the absence of salt is very pronounced.

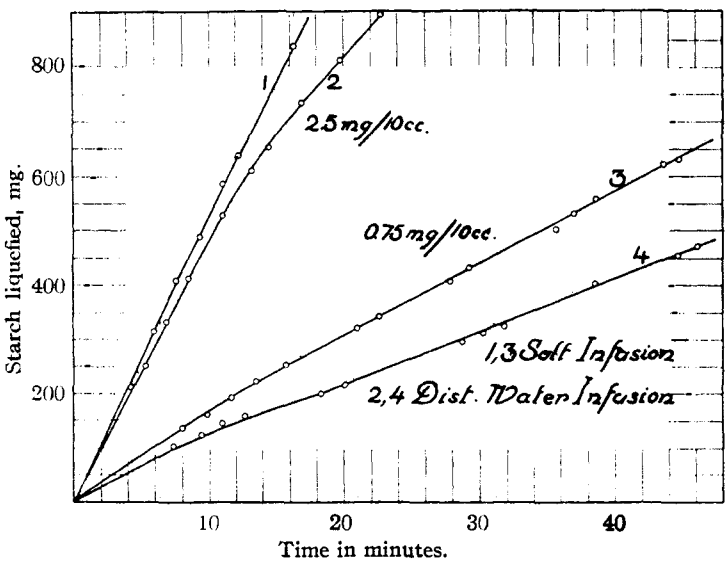

Fig. 2.—Rate curve for α-amylase.

These data were plotted on a large scale as before and the initial slopes measured. The rates at zero time were directly proportional to the enzyme concentration as in the case of the distilled water infusions. The fact that the initial rates for distilled water infusions are proportional to enzyme concentration again indicates that the start of the reaction is the best possible time for measurements of enzyme concentrations. Adsorption effects, etc., have the least influence at zero time. The data for salt infusions is shown in Table V.

The higher initial rates for salt infusions may be due to desorption of the enzyme from protein bodies in the infusion.

The results for both types of infusions are plotted in Fig. 3. The straight lines obtained are a good indication that the Michaelis theory applies to alpha-amylase. Hanes has shown that beta-amylase conforms to the Michaelis theory. He has studied[4] the saccharifying action at the start of the reaction in a similar manner to our study of alpha-amylase. Our method was perfected before the publication of Hanes' results.

TABLE V

Infusion concn., mg. per 10 cc.	7	5	2.5	1.25	0.75
Liquefaction of starch at zero time, mg. per min.	165	115	56	28	17

As stated in the introduction the proportionality between initial rate of action and enzyme concentration offers a logical and rational means

(3) S. Jozsa and H. C. Gore, *Ind. Eng. Chem.*, **24**, 95 (1932).

(4) C. S. Hanes, *Biochem. J.*, **26**, 1406 (1932).

of estimating alpha-amylase concentrations. The choice of the enzyme unit is arbitrary in so far as its magnitude is concerned. Our unit of 25 mg. of starch per minute at zero time at 21° was chosen to conform numerically with a previous method of expression used in our laboratories. The temperature of 21° was chosen in order to conform with the Lintner determination of β-amylase. The determination of the number of liquefons per gram accurately estimates concentration of enzyme as well as liquefying power at zero time as emphasized above.

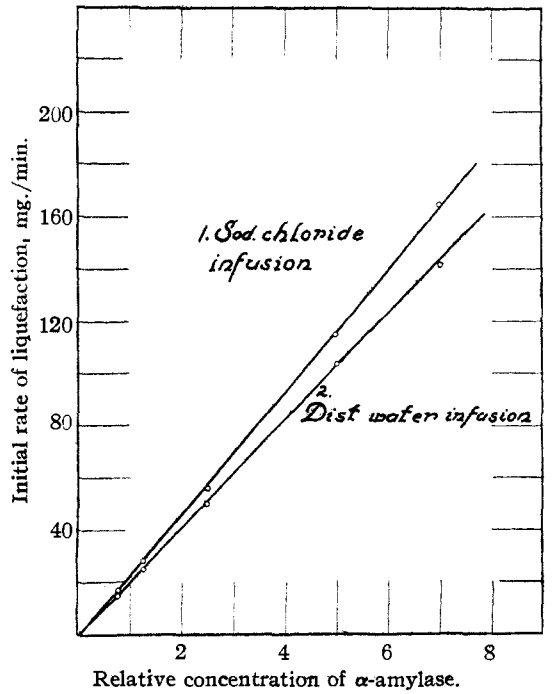

Fig. 3.—Rate-concentration curves.

If it were necessary to plot a rate curve for every determination, the enzyme unit would be of little value; but we have been able to derive an empirical relation between the number of liquefons present per 10 cc. of infusion and the number of milligrams of dry starch liquefied in one hour under the specified conditions. This relationship enables us to apply the enzyme unit, or liquefon, to the determination of alpha-amylase. The experimental data showing the relation between the number of liquefons per 10 cc. acting on the paste and the number of milligrams of dry starch liquefied are given in Table VI. The measurements were made by using the pipet method as described in the paper on the determination of alpha-amylase. These data are plotted in Fig. 4, where

TABLE VI

Liquefons per 10 cc.	$\log_{10}$ liquefons	Starch liquefied, mg.
0.68	−0.166	793
.91	− .041	1000
1.37	+ .135	1301
1.82	.260	1534
2.28	.357	1712
2.71	.433	1859
3.64	.561	2066
4.10	.612	2145
4.55	.658	2247
5.46	.737	2372

the logarithmic relation between the variables is very evident. This relation holds strictly, however, only for the experimental range considered (20–60% of starch liquefied). The range is large and it is very easy to make liquefon determinations for enzyme preparations of widely varying strengths. It is possible to measure accurately any preparation by using the proper dilution and the proper concentration of sodium chloride as an "activator."

The liquefon–starch curve was fitted by an empirical equation of the form: $\log_{10} L = (S - 1078)\ (0.000565)$, in which L = liquefons per 10 cc.; S = milligrams of starch liquefied in one hour.

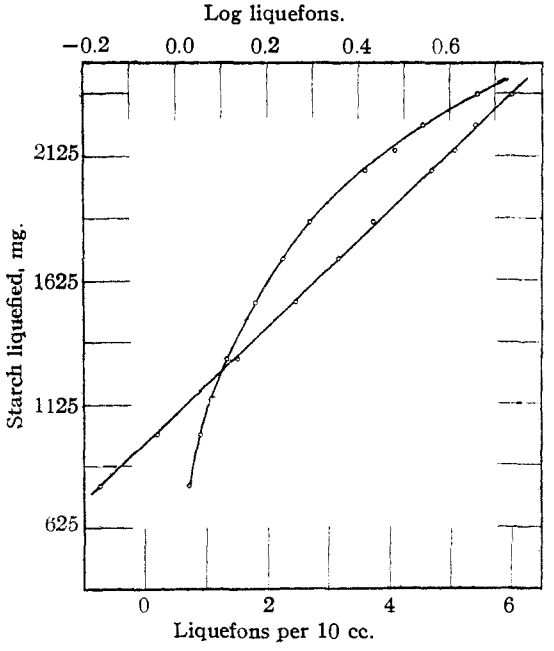

Fig. 4.—Starch–liquefon functions.

The curve was fitted in the usual manner by plotting the $\log_{10} L$ against S and then using the method of least squares to find the best straight line through the points.

This equation is applicable over the experimental range with an error of about 1–2%. The table presented in the paper on the determination of alpha-amylase was compiled with the aid of this equation.

It is evident that the equation should be applicable to other enzyme preparations such as taka-diastase, pancreatin, etc., so long as the rate curves of these preparations were the same as those of malt amylase. This has not been demonstrated as yet but it is probable that when each preparation is properly treated with sodium chloride as a desorption or activating agent the rate curves would follow closely those of malt amylase. If the rate curves are totally different it is only necessary that we change the empirical expression to fit the preparation if great accuracy is desired.

The enzyme-unit method is generally applicable in enzyme studies. It has been applied to the study of invertase with excellent results which will soon be published. Work is now in progress on units for β-amylase and for proteolytic enzymes such as papain.

The authors wish to express their appreciation to Dr. C. N. Frey and Mr. Q. Landis for valuable suggestions in carrying out this investigation.

Summary

Experimental evidence has been presented which permits the definition and use of a rational amylase unit termed the liquefon. Its use in the determination of alpha-amylase has been described.

NEW YORK CITY RECEIVED OCTOBER 23, 1934

[CONTRIBUTION NO. 26 FROM THE DEPARTMENT OF CHEMISTRY OF THE POLYTECHNIC INSTITUTE OF BROOKLYN]

Nitroguanylhydrazones of Some Common Aldehydes and Ketones[1]

BY W. F. WHITMORE, A. J. REVUKAS AND G. B. L. SMITH

Phillips and Williams[2] have reported that nitroaminoguanidine reacts with certain aldehydes and ketones to form crystalline substances. These investigators suggested that the reaction was probably that characteristic of carbonyl groups and that nitroguanylhydrazones were formed. This postulation is supported by the work of Thiele,[3] who found that aminoguanidine formed a guanylhydrazone with benzaldehyde. Woods[4] confirmed the observations of Phillips and Williams and studied in some detail the crystallographic and optical properties of certain of the nitroguanylhydrazones.[5] This paper describes the methods of preparation, analyses and some physical properties of a series of nitroguanylhydrazones of some common aldehydes and ketones. It is hoped that these and future data will establish the usefulness of nitroaminoguanidine as a reagent for the identification of aldehydes and ketones.

(1) An abstract of part of the thesis submitted to the Faculty of the Polytechnic Institute of Brooklyn in June, 1934, by Mr. Revukas in partial fulfilment of the requirements for the degree of Master of Science in Chemistry.

(2) Phillips and Williams, THIS JOURNAL, 50, 2465 (1928).

(3) Thiele, *Ann.*, 270, 35 (1892).

(4) Woods, Thesis, M.S. in Chemistry, Polytechnic Institute of Brooklyn, 1932.

(5) These studies are being extended and will be reported in a future communication.

Experimental Part

Materials.—Nitroaminoguanidine ($H_2NNHC(=NH)NHNO_2$) was prepared by the method described by Phillips and Williams.[2] The aldehydes and ketones were purchased from Schering–Kahlbaum and were the "pure" grade.

General Procedure.—One to five drops of the pure aldehyde or ketone (if liquid), or approximately 0.05 g. (if solid) is dissolved in one ml. of water or if not water-soluble in one ml. of acetone-free methyl alcohol. To this solution is added 5 ml. of an aqueous solution of nitroaminoguanidine saturated at 70° (approximately 0.15 g. of nitroaminoguanidine) together with 0.25 ml. of glacial acetic acid. If precipitation does not take place within five minutes the solution is cooled in an ice-bath. The crystalline product is separated by filtration and recrystallized. The recrystallization medium is either hot water, hot 50% methyl alcohol, or hot absolute methyl alcohol, depending upon the solubility of the respective nitroguanylhydrazones. In general, the water-soluble aldehydes and ketones yield nitroguanylhydrazones which may be readily recrystallized from water, and those insoluble in water must be recrystallized from methyl alcohol. Precipitation occurs when the concentration of the aldehyde or