2. These red respiratory proteins form together with hemoglobin, which is strictly limited to the five higher classes of the vertebrates, a system of molecules built up of units of weight 34,500 (and $^1/_2 \times 34{,}500$) in simple proportions.

UPSALA, SWEDEN RECEIVED JANUARY 6, 1934

[CONTRIBUTION FROM THE LABORATORY OF PHYSICAL CHEMISTRY OF THE UNIVERSITY OF UPSALA]

The Ultracentrifugal Study of Pomelin

BY LAURA KREJCI[1] AND THE SVEDBERG

In two recent communications Saunders[2] has reported the results of an investigation concerning the amount of nitrogen extracted from orange seed meal by solutions of different salts. He has found that with one exception, and that a logical one, the amounts extracted are identical regardless of the salt used as extracting agent. This circumstance is of especial interest in view of Gortner's findings[3] that wheat flour yields widely different amounts of nitrogen on extraction with solutions of various salts; the potassium halides, in particular, show a marked lyotropic series. Saunders has suggested that this difference in behavior may be a consequence of the fact that the orange seed protein, pomelin, is a well-defined crystalline globulin, while wheat flour contains a complex mixture of proteins and might therefore be expected to yield varying amounts of nitrogen on treatment with different salts. Since, however, solutions of different salts in varying concentration have been found to extract different amounts of protein even from seed material which is known to contain crystalline globulin,[4] and since it is also possible that different salts may extract the same amounts of nitrogen from a meal and yet not extract a chemical entity, it seemed to be of interest to make an ultracentrifugal study of the orange seed globulin in order to determine more precisely than would otherwise be possible the character of the material extracted by the salts from the orange seed meal. At the suggestion of Dr. Saunders such a study was undertaken in this Laboratory.

Material Used

Dr. Saunders was kind enough to provide the material for the investigation, both orange seed meal and a sample of dried pomelin. The orange seed meal had been prepared in the same manner as for the extraction studies. The seeds were ground in a mill, extracted repeatedly with benzene to remove the oil, air-dried and sifted. The dried pomelin had been prepared by the ammonium sulfate-dialysis method described by Saunders,[5] and dried with alcohol and ether.

The dried pomelin was rejected in favor of the orange seed meal as the source of protein for use in the ultracentrifuge because of the possibility that the alcohol and ether used during the preparation might have caused denaturation. However, near the completion of the study two runs were made with solutions of the dried pomelin in order to determine the effect if any of the preparation process on the protein.

Comparison of Chloride, Bromide and Iodide Extracts. Sedimentation Velocity Measurements.—In order to make a comparison of the protein matter extracted by the different salts, portions of the orange seed meal were extracted with half normal solutions of potassium chloride, potassium bromide and potassium iodide. After the treatment described below, the extracts were studied in the ultracentrifuge by the sedimentation velocity method. The results are listed in Table I.[6]

TABLE I

COMPARISON OF EXTRACTS OF ORANGE SEED MEAL. SEDIMENTATION VELOCITY MEASUREMENTS

Centrifugal force about 100,000 times gravity; thickness of column of solution, 1.2 cm.; source of light, mercury lamp; light filter, chlorine and bromine; plates, Imperial Process; exposure time, one minute; metol hydroquinone developer, one minute. Solvent in all cases: 0.5 N KCl.

Description of treatment	$s_{20} \times 10^{13}$								
	KCl extract			KBr extract			KI extract		
Pptd. once with $(NH_4)_2SO_4$	..	..	..	..	11.6	..	..	..	..
Pptd. 3 times with $(NH_4)_2SO_4$	..	11.3	..	..	..	..	2.8	11.4	..
Pptd. 4 times with $(NH_4)_2SO_4$	1.5	11.2	..	..	..	..	..	..	..
Fraction pptd. by dilution	..	11.8	16.9	..	12.0	16.7	..	11.3	17.0
Fraction not pptd. by diln.	0.8	11.7	..	1.5	..	..	1.7	..	..
Second extract of meal, pptd. once with $(NH_4)_2SO_4$	..	11.0	..	..	..	..	..	..	..

(1) Fellow of the American Scandinavian Foundation.

(2) Saunders, THIS JOURNAL, **53**, 696 (1931); Rotha and Saunders, *ibid.*, **54**, 342 (1932).

(3) Gortner, Hoffman and Sinclair, "Colloid Symposium Monograph," The Chemical Catalog Co., Inc., New York, 1928, Vol. V, p. 187.

(4) Osborne and Harris, *Am. J. Physiol.*, **14**, 151 (1905).

(5) Saunders, Ref. 2, p. 697.

(6) In all cases the sedimentation constant has been corrected for the density and viscosity of the salt present to a basis of sedimentation in pure water. See Svedberg and Stamm, THIS JOURNAL, **51**, 2177 (1929).

The crude extracts were found to contain so large a content of non-centrifugible light-absorbing material that study in the ultracentrifuge was impossible without previous purification. Each extract was saturated with solid ammonium sulfate; the precipitated protein was washed on the filter with a saturated solution of the same salt, then dissolved in half normal potassium chloride. This procedure was repeated twice more on the potassium iodide extract in order to remove the iodide as completely as possible because of its high light absorption; the chloride extract was also precipitated twice more as a check. In the ultracentrifuge all three solutions showed the presence of non-centrifugible material, still in comparatively high concentration, but lower in the chloride and iodide extracts than in the bromide extract; a fairly sharp sedimentation boundary with a sedimentation constant of about 11; and some slowly sedimenting material the boundary for which was difficult to measure. Except for the differences in the content of the light-absorbing contaminant, all these solutions appeared to be identical, though the presence of this contaminant made an accurate comparison difficult.

The chloride extract was precipitated a fourth time; the non-centrifugible contaminant still persisted. Precipitation by ammonium sulfate as a means of eliminating this material was therefore abandoned in favor of precipitation by dilution. The chloride extract was diluted with distilled water to seven times its volume, the bromide extract to fourteen times its volume, and the iodide extract to eight times its volume. The precipitated protein was suspended in half normal potassium chloride; the undissolved protein was removed by centrifuging. In the ultracentrifuge all three solutions proved to be absolutely free from non-centrifugible material. The boundary of sedimentation constant 11 was sharply defined, and there was a second boundary of sedimentation constant 16, which had not been detected in the previous runs. The bromide and iodide extracts both contained traces of the slowly sedimenting material; the chloride extract contained none at all.

The diluted solutions from which the heavier fractions had been precipitated were saturated with ammonium sulfate. In each case a small amount of precipitate was formed; this was collected on a filter and dissolved in 0.5 *N* potassium chloride. In the ultracentrifuge each was found to consist of the slowly sedimenting material and non-centrifugible matter. The chloride extract contained in addition sufficient material of sedimentation constant 11 to permit measurement of the boundary; the bromide and iodide extracts contained none.

All evidence seems to indicate that the three extracts were identical in content. All three components were present in each extract, and so far as could be ascertained they were present in about the same proportions. At least no such decided differences were apparent as have been found to exist between the chloride, bromide and iodide extracts of wheat flour.[7]

The residue from the extraction of the orange seed meal with potassium chloride was extracted a second time with the same solvent. The solution was subjected to a single precipitation with ammonium sulfate. Except for the lower concentration of protein it seemed identical with the bromide extract after similar treatment. There was no evidence that successive extractions of the meal might yield different mixtures of protein. Investigation of this fraction was therefore discontinued.

In view of the identity of the fractions extracted by the different salts, parallel study of the three extracts was also discontinued, and the experimental work which is now to be described was limited, except for one determination, to the fraction of the bromide extract which had been precipitated by dilution.

Further Study of Pomelin Solutions. Denaturation.—Further study centered about the effect of time of standing on the protein precipitated by the dilution of the pomelin solutions. The results are summarized in Table II.

TABLE II

POMELIN. SEDIMENTATION VELOCITY MEASUREMENTS

Experimental conditions as in Table I

Solution	Solvent, *N*	$s_{20} \times 10^{13}$		
1	KCl 0.5	12.0	16.7	
2	KCl 0.5	12.3	16.0	
3	KCl 0.5	11.8	17.1	
4	KCl 0.5	12.4	17.4	
5	KCl 1.0	12.9	..	28.2
6	KCl 1.0	..	..	21.8
7	KCl 0.5	11.3	16.6	
8	KCl 0.5	12.2	16.6	

The fraction of the bromide extract which had been precipitated by dilution (solution 1) was in turn diluted with five times its volume of water and allowed to stand at room temperature for one hour. The precipitated protein was removed from the solution and suspended in 0.5 *N* potassium chloride; this constitutes solution 2. The diluted solution 1 was then placed in a refrigerator and allowed to stand for a week longer. During this time a considerable additional quantity of protein was precipitated. It was removed from the solution and suspended in 0.5 *N* potassium chloride; this constitutes solution 3. Both were studied in the ultracentrifuge. Solution 2 contained a slightly higher percentage of the component of sedimentation constant 16 than solution 1 from which it had been prepared; solution 3 contained a lower percentage, but contained in addition a small amount of inhomogeneous material of still higher sedimentation constant.

Small amounts of insoluble residue were obtained in the preparation of solutions 2 and 3. Similar residues had been obtained in the preparation of solution 1 and the corresponding fractions of the chloride and iodide extracts. The fact that the volume of potassium chloride solution used was in each case less than the volume of solution from which the protein had been precipitated was doubtless in part responsible. But the possibility of denaturation with the formation of proteans, and especially the detection of heavy inhomogeneous material in solution 3, made it seem advisable to examine these residues.

The residue from the preparation of solution 1 was suspended in normal potassium chloride (solution 1a); the residue from the corresponding fraction of the iodide extract (solution 7) was suspended in half normal potas-

(7) Svedberg and Krejci, "The Ultracentrifugal Study of the Proteins of Wheat Flour. I. The Salt Extractable Proteins." Communicated to *Cereal Chem.*

sium chloride (solution 8). Some of the material went into solution, but much remained undissolved. The undissolved material was discarded.

Solution 8 contained sufficient protein matter to permit study in the ultracentrifuge. So far as could be detected, it was identical with solution 7.

Solution 1a was diluted with five times its volume of water and allowed to stand for five hours. The precipitated protein was removed and suspended in 0.5 *N* potassium chloride solution; this constitutes solution 4. This solution was found to contain the same two components as solution 1, but the concentration of the component of sedimentation constant 16 was much greater; furthermore, there was evidence of the heavier inhomogeneous material which had been detected in solution 3.

The residue resulting from the preparation of solution 4 was suspended in normal potassium chloride solution; this constitutes solution 5. The component of sedimentation constant 11 was still present in small degree; but the bulk of the protein consisted of heavy inhomogeneous material of average sedimentation 28. The component of sedimentation constant 16, if present at all, was merged with the heavy material.

The undissolved material was treated with a further quantity of normal potassium chloride solution; this constitutes solution 6. The protein contained in it consisted entirely of inhomogeneous matter; the average sedimentation constant was about 22.

These results seem to indicate that pomelin is readily denatured in solutions of low salt content, and that one of the steps in this denaturation process is the formation of the aggregates which constitute the inhomogeneous material detected in the determinations described above.

*P*H Stability Range of Pomelin

Determinations of the sedimentation constant of pomelin were made in the *P*H range 3.3–12.9. The material used for these determinations was prepared in the following manner.

Ten grams of orange seed meal was extracted with 100 cc. of normal potassium chloride solution. The solution was set to dialyze in collodion bags against water for the first day, thereafter against frequently renewed 0.1 *N* potassium chloride. Fifty hours later the material was removed from the bags. The precipitated protein was washed three times with 0.1 *N* potassium chloride, then suspended in 50 cc. of 0.5 *N* potassium chloride; twelve hours later the undissolved material was removed. This solution was diluted with 200 cc. of water. The precipitate was suspended in 15 cc. of 0.5 *N* potassium chloride, and the undissolved protein was removed several hours later.

This solution, which consisted chiefly of the component of sedimentation constant 11 with small amounts of the slowly sedimenting material and the component of sedimentation constant 16, was used as the stock solution for the determination of the stability range of pomelin. A small portion was diluted with the appropriate buffer just before use in the ultracentrifuge. The determinations are summarized in Table III.

The low solubility of pomelin in the *P*H range 3.3–4.6 made impossible the ultracentrifugal study of the protein remaining in solution; determinations were limited to the

TABLE III

POMELIN. SEDIMENTATION VELOCITY MEASUREMENTS

Centrifugal force about 200,000 times gravity; exposure time 15–60 sec.; other experimental conditions as in Table I

*P*H of soln.	Solvent *M*	*M*	*M*	$s_{20} \times 10^{13}$
3.3	KCl 0.5	HCl 0.0035		[a,b,c]
3.5	KCl .5	HCl .0009		[a,b,d]
3.5 ppt. at 6.8	KCl .5	Na_2HPO_4 .050	KH_2PO_4 0.050	11.1
3.7	KCl .5	HAc .036	NaAc .004	[a,b]
3.7 ppt. at 6.8	KCl .5	Na_2HPO_4 .050	KH_2PO_4 .050	11.3
4.0	KCl .5	HAc .042	NaAc .008	[a,b]
4.0 ppt. at 6.8	KCl .5	Na_2HPO_4 .050	KH_2PO_4 .050	11.4
4.2	KCl .5	HAc .021	NaAc .014	[a,b]
4.2 ppt. at 6.8	KCl .5	Na_2HPO_4 .125	KH_2PO_4 .125	11.2
4.6	KCl .5	Na_2HPO_4 .001	KH_2PO_4 .073	11.5
4.6	KCl .5	Na_2HPO_4 .001	KH_2PO_4 .073	11.7
4.6 ppt. at 6.8	KCl .5	Na_2HPO_4 .083	KH_2PO_4 .083	11.2
4.8	KCl .5	HAc .032	NaAc .048	12.9
5.1	KCl .5	HAc .005	NaAc .020	11.8
6.3	KCl .5	Na_2HPO_4 .014	KH_2PO_4 .044	11.8
10.1	KCl .5	Na_2HPO_4 .018	NaOH .003	11.8
10.3	KCl .5	Na_2HPO_4 .078	NaOH .013	10.9
10.9	KCl .5	Na_2HPO_4 .060	NaOH .020	11.1
12.6	KCl .5	Na_2HPO_4 .016	NaOH .064	3.2 11.9
12.9	KCl .5	Na_2HPO_4 .003	NaOH .083	2.6 10.5
12.9	KCl .5	Na_2HPO_4 .002	NaOH .041	3.1 11.9
12.9 → 6.8	KCl .5	Na_2HPO_4 .050	NaOH .050	12.1

[a] Protein precipitated. [b] Protein content too low for determination of sedimentation velocity. [c] Denaturation complete. [d] Partial denaturation.

protein precipitated under these conditions, redissolved in neutral buffers.[8]

The results indicate that the component of sedimentation constant 11 is stable from *P*H 3.5 to about *P*H 12. At *P*H 3.5 acid denaturation has already begun; but the portion of the precipitated protein which can still be dissolved in neutral buffers retains its original sedimentation constant unchanged. At *P*H 12.6 and beyond there is a gradual decrease in the content of component 11 and an accompanying increase both in the content of slowly sedimenting material and in the light absorption; this indicates clearly that the molecules are being broken up into lighter fragments. The portion of component 11 which persists at these alkalinities is unchanged so far as sedimentation constant is concerned. The decomposition products are not identical with the slowly sedimenting material originally present in the solution; this was demonstrated by the following observation. Solution *P*H 12.9 was brought to *P*H 6.8 by dialysis. A portion of the protein was precipitated, but that which remained in solution consisted of the slowly sedimenting material in the amount which had originally been present, and of component 11 in the amount present at *P*H 12.9. The precipitated protein must therefore have been an aggregation product formed from the fragments of the component of sedimentation constant 11.

A second solution, which was obtained from the protein remaining from the preparation of the first stock solution, and which contained higher percentages of the component of sedimentation constant 16 and the slowly sedimenting material, was also studied under different conditions of *P*H. The results are summarized in Table IV.

either of the other components. It was apparently unchanged both at 3.3 and at 12.9.

In the *P*H range where low solubility caused precipitation of protein, the precipitate was always found to be richer in component 16 and poorer in slowly sedimenting material than the original solution.

Dried Pomelin.—Two determinations were made with protein extracted from the sample of dried pomelin furnished by Dr. Saunders.

Twelve cc. of 0.5 *N* potassium chloride which was also *M*/20 in Na_2HPO_4 was added to 0.12 g. of dried pomelin. After standing overnight, the mixture was centrifuged to remove the undissolved protein. The solution was used full strength in the ultracentrifuge. The results are given in Table V.

The material contained, in addition to the component of sedimentation constant 11 and the slowly sedimenting material, a boundary of sedimentation constant 7–8 which had not been met with in any of the work on the protein freshly extracted from the orange seed meal; there was no trace of the component of sedimentation constant 16. The run at higher speed, which was intended to effect a more complete resolution of the three boundaries, actually did just the reverse—spread each until all three were merged. This is clear indication that each represented an inhomogeneous mixture the components of which were separated more rapidly in the higher centrifugal fields. The inhomogeneity must have been the result of decomposition. It is possible that this decomposition may have occurred in a manner similar to that observed in alkaline decomposition: the breaking up of the larger molecules into fragments, and the aggregation of these fragments into larger particles.

TABLE IV

POMELIN. SEDIMENTATION VELOCITY MEASUREMENTS

Experimental conditions as in Table I

*P*H of soln.	Solvent *M*					$s_{20} \times 10^{13}$		
	M	*M*		*M*				
3.3	KCl 0.5	HAc	0.045	NaAc	0.005		a	
5.0	KCl .5	Na_2HPO_4	.002	KH_2PO_4	.053		11.1[b]	
5.0 ppt. at 6.8	KCl .5	Na_2HPO_4	.008	KH_2PO_4	.008		12.7	17.3
5.3	KCl .5	HAc	.007	NaAc	.063		11.7[b]	
5.6	KCl .5	Na_2HPO_4	.003	KH_2PO_4	.042		11.5[b]	
6.2	KCl .5	Na_2HPO_4	.013	KH_2PO_4	.054		11.1	17.5
6.8	KCl .5	Na_2HPO_4	.025	KH_2PO_4	.025		11.3	16.0
8.0	KCl .5	Na_2HPO_4	.063	KH_2PO_4	.004		11.2	16.4
10.3	KCl .5	Na_2HPO_4	.057	NaOH	.009		10.9	
12.9	KCl .5	Na_2HPO_4	.002	NaOH	.041	3.1	11.9	

[a] Denaturation complete. [b] Some protein precipitated.

Component 16 is less stable in the presence of alkali than component 11. Even at *P*H 10.3 there is an evident decrease in the concentration of component 16 and an increase both in light absorption and content of slowly sedimenting material, and at *P*H 12.9 it has completely disappeared. In the acid range denaturation is complete at *P*H 3.3, but no determination was made of the exact point at which denaturation begins.

The slowly sedimenting material is more stable than

TABLE V

DRIED POMELIN. SEDIMENTATION VELOCITY MEASUREMENTS

Thickness of column of solution 0.8 cm.; plates, Hauff Röntgen; time of exposure, 25–30 sec.; other experimental conditions as in Table I.

Solvent KCl, *N*	Solvent Na_2HPO_4, *M*	*P*H of soln.	Centrifugal force × g.	$s_{20} \times 10^{13}$		
0.5	0.05	8.0	200,000	..	8.0	11.9
.5	.05	8.0	250,000	2.8	6.9	10.5

(8) This low solubility is undoubtedly the explanation for the small amount of nitrogen extracted from orange seed meal by solutions of sodium dihydrogen phosphate, as reported by Rotha and Saunders [THIS JOURNAL, 54, 344 (1932)].

Discussion of Results

The results of this investigation do not support the hypothesis that salt solutions extract a definite chemical entity from orange seed meal. Three molecular species were found in every extract of the meal, regardless of the salt used as extracting agent. So far as could be determined, however, the proportions of the three species were the same in every instance. Any attempt to explain this behavior would necessitate an involved study of solubility relationships.

The average value for the sedimentation constant of the chief constituent of pomelin is 11.38×10^{-13}. This agrees well with those found for the other seed proteins which have been studied in this Laboratory. The comparison is given in Table VI. An attempt was made to determine the molecular weight by means of sedimentation equilibrium measurements. The results were inconclusive because of the presence of both the heavier and lighter components in the material used. However, the drift from about 200,000 at the meniscus to 300,000 at the bottom of the cell may perhaps be interpreted to indicate that the molecular weight lies in the neighborhood of 210,000, the value found for the other seed proteins.[9]

TABLE VI

COMPARISON OF SEED PROTEINS

Protein	Source	$s_{20} \times 10^{13}$
Edestin	Hemp seed meal	12.8[a]
Amandin	Almond meal	11.41[b]
Excelsin	Brazil nut meal	11.87[b]
Legumin	Vetch flour	11.48[c]
Pomelin	Orange seed meal	11.38

[a] Svedberg and Stamm, THIS JOURNAL, 51, 2170 (1929).

[b] Svedberg and Sjögren, *ibid.*, 52, 279 (1930).

[c] Sjögren and Svedberg, *ibid.*, 52, 3279 (1930).

The constant for the slowly sedimenting material was difficult to measure and was calculated only in the most favorable instances. The average value is 2.2×10^{-13}.

The average value for the sedimentation constant of the heavy constituent is 16.75×10^{-13}. It is not impossible that this heavy species may be a denaturation product of the chief constituent. On no occasion was it detected until after dilution or dialysis, and prolonged contact of the protein with solutions of low salt content has been shown to result in the formation of still heavier aggregation products. A sedimentation constant of this magnitude has been met with frequently in the study of the respiratory proteins of the crustacea; the mean value is 16.9×10^{-13}.[10] The molecules possessing this constant have a weight of about 320,000, which is approximately three-halves that of the predominant pomelin component.

It seems unlikely that Saunders' preparation of pomelin contained a single chemical entity, the constituent of sedimentation constant 11, even before the drying with alcohol and ether. Undoubtedly some slowly sedimenting matter was present, because both saturation with ammonium sulfate and dilution or dialysis have been shown to precipitate out this material. The heavy constituent must almost certainly have been present also, in spite of the fact that no trace of it was found in the dried material. The procedure followed in the parallel study of the chloride, bromide and iodide extracts of orange seed meal differed only slightly from Saunders' procedure; yet in each case the heavy constituent was present in good proportion.

The dilution method employed by Carpenter and Lovelace[11] seems likely, in the light of the present investigation, to give a more homogeneous product. Normal sodium chloride solution was used for extraction of the meal. The protein was twice precipitated by dilution, the first time with five volumes, the second with ten volumes, of water; the solutions were filtered through asbestos and paper pulp immediately before and after each dilution. The comparatively high salt content of the diluted solutions should preclude contamination with the slowly sedimenting material, and the removal of the precipitate first formed on dilution should tend to decrease the content of the heavy species. Furthermore, if this heavy species actually is a denaturation product, the tendency to form should be depressed by the high salt content.

Carpenter and Lovelace report that the isoelectric point of pomelin is 5.23. This is in qualitative agreement with the low solubility which was observed from P_H 5.6 continuously through to the acid denaturation point, P_H 3.3.

The expenses connected with this investigation have been defrayed by grants from the Rockefeller Foundation, the Nobel Foundation and the foundation "Therese och Johann Anderssons Minne."

(9) Svedberg and co-workers, a, b, c references Table VI.

(10) Svedberg, *J. Biol. Chem.*, **103**, 320 (1933).

(11) Carpenter and Lovelace, THIS JOURNAL, **55**, 3738 (1933).

Summary

1. An ultracentrifugal study has been made of the orange seed globulin, pomelin.

2. Pomelin was found to consist of three components, the sedimentation constants of which are 2.2, 11.38 and 16.75×10^{-13}.

3. Half-normal solutions of potassium chloride, potassium bromide and potassium iodide were found to extract the same relative amounts of the three components.

4. The component of sedimentation constant 11.38, the chief constituent of pomelin, was found to be stable throughout the *P*H range 3.5–12. Acid denaturation is complete at *P*H 3.3. In the alkaline region beyond *P*H 12 the molecules are broken up into smaller fragments which coalesce to a coherent precipitate when the *P*H of the solution is brought back to the neutral range. The component of sedimentation constant 16.75 begins to decompose at *P*H 10.3 in the alkaline region. Acid denaturation is complete at *P*H 3.3.

5. Pomelin was found to be readily denatured in solutions of low salt content.

6. A sample of dried pomelin gave evidence of denaturation.

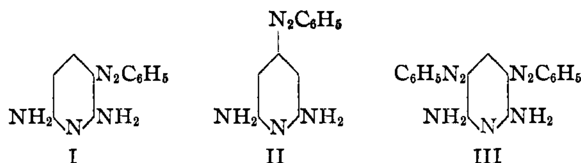
UPSALA, SWEDEN RECEIVED FEBRUARY 13, 1934

[CONTRIBUTION FROM THE CHEMICAL LABORATORY, ACADEMY OF SCIENCES U. R. S. S., MOSCOW]

γ-Phenylazo-α,α'-diaminopyridine

BY A. E. CHICHIBABIN AND E. D. OSSETROWA

Ivan Ostromislensky[1] by coupling diazotized aniline with α,α'-diaminopyridine obtained as the chief product β-phenylazo-α,α'-diaminopyridine (formula I). This substance had been obtained much earlier in the same way by Chichibabin and Zeide.[2] In addition to this main product Ostromislensky mentions two other substances to which he ascribes the formulas II and III.



There is nothing improbable in the formation of a substance having formula III. It is, however, highly improbable that a substance having formula II should be formed under these circumstances because so far as we know there is not a single instance among the prodigious number of coupling reactions which have been investigated where the azo group enters the meta position. Moreover, the orienting influences would make such an entry particularly improbable in the pyridine series.

In this situation we have undertaken to prepare a substance having formula II by a series of unambiguous reactions, as follows.

γ-Chlorodipicolinic acid IV was treated with phenylhydrazine to form γ-phenylhydrazodipicolinic acid V. This was oxidized by atmospheric oxygen to give γ-phenylazodipicolinic acid VI. The methyl ester of this acid was treated with hydrazine, which served to reduce the azo group. The γ-phenylhydrazo-α,α'-dihydrazidopyridine VII formed was oxidized by atmospheric oxygen to give γ-phenylazo-α,α'-dihydrazidocarbopyridine VIII. This was treated with nitrous acid to form γ-phenylazo-α,α'-dihydrazidocarbopyridine IX, which was converted to the diurethan X by boiling in alcoholic solution. If the boiling is not continued for a sufficient length of time the monourethan XI may also be obtained. The saponification of the diurethan X gives the desired γ-phenylazo-α,α'-diaminopyridine II. The properties of this substance differ from those indicated by Ostromislensky for the substance II.

Numerous experiments were made in our laboratory on the action of diazotized aniline on α,α'-diaminopyridine. With an excess of diazotized aniline the compound III is formed, together with other products, and has the properties of the compound described as such in the patent. Numerous attempts to prepare III without an excess of diazotized aniline indicated that it either is not formed at all or is formed only in an insignificant quantity. β-Phenylazo-α,α'-diaminopyridine may be obtained quite pure from one crystallization. In spite of numerous prepara-

(1) U. S. Patent 1,680,109 (Serial No. 97,771); *Chem. Zentr.*, I, 1026 (1929).

(2) Chichibabin and Zeide, *J. Russ. Phys.-Chem. Soc.*, **46**, 1216 (1914); *Chem. Zentr.*, I, 2, 1069 (1915).