pounds. It is illustrated below in the preparation of 4-nitro-2,4,6-trimethyl-2,6-diazaheptane.

Method B. Preparation of 4-Nitro-2,4,6-trimethyl-2,6-diazaheptane from 2-Nitro-2-methyl-1,3-propanediol and Dimethylamine.—A mixture of 351 g. of a 25.7% dimethylamine solution (2 moles) and 135 g. of 2-nitro-2-methyl-1,3-propanediol (1 mole) was placed in a quart bottle and left in the refrigerator overnight. A reaction took place at once and a non-aqueous layer separated. This layer solidified on cooling, weight 136.4 g. It liquefied at room temperature and was dried over anhydrous sodium sulfate. Crude conversion was 72%. Attempted distillation of a portion of the product resulted in decomposition; m. p. of crystals, 32.0°. The remainder of the material was reduced and identified as the amine.

III. Hydrogenation of Nitro Amines to Polyamines[2]

The various nitro amines that were prepared were in turn hydrogenated to the corresponding polyamines. The method of reduction is illustrated in the following example:

One mole (146 g.) of N-(2-nitroisobutyl)-dimethylamine was dissolved in 500 ml. of methanol and 8 g. of Raney nickel catalyst added. The reduction was carried out at 30–50° under 500 lb. sq. in. of hydrogen. The completeness of the reduction may be followed by the absorption of hydrogen. The solution was filtered on removal from the bomb. The filtrate was distilled at room temperature to remove methanol. When nearly all the alcohol was removed, the distillation was stopped and 200 ml. of benzene added. A Dean and Stark moisture trap[3] was connected to the column and the mixture refluxed to remove water. (A binary constant boiling mixture of methanol and benzene distilled first.) Distillation of the residual product and benzene was then resumed, yielding 8 g. of material, b. p. 80–115° and 77 g. at 119° of the N-(2-aminoisobutyl)-dimethylamine. The residue was 5 g., conversion 66.5%.

The conversion to pure polyamines, on the basis of the nitroparaffins, was the same whether the pure nitro amines or the crude nitro amines were hydrogenated.

Acknowledgment.—The author wishes to thank Dr. J. A. Riddick, Dr. P. C. Markunas and associates for the analyses reported in this work.

Summary

The reaction of secondary aliphatic amines with formaldehyde and both primary and secondary nitroparaffins has been demonstrated. Two methods have been used to prepare the nitro amines either of which gives the same end-product, *e. g.*, the amine plus formaldehyde when treated with the nitroparaffin gives the nitro amine or the amine may be added directly to the nitro alcohol (or diol) to give the nitro amine. The reactions are illustrated as follows

$$R_2NH + CH_2O + (CH_3)_2CHNO_2 \longrightarrow R_2NCH_2C(CH_3)_2NO_2 + H_2O$$

$$2R_2NH + CH_2C(NO_2)(CH_2OH)_2 \longrightarrow R_2NHC_2\text{—}C(CH_3)(NO_2)\text{—}CH_2NR_2 + 2H_2O$$

The nitro amines were reduced to the corresponding polyamines using Raney nickel catalyst.

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(2) It is pointed out that hydrogenation of nitro amines to polyamines should not be started at high temperatures since a violent reaction may take place because of the exothermic nature of the hydrogenation process.

(3) Dean and Stark, *Ind. Eng. Chem.*, **12**, 486 (1920).

[CONTRIBUTION FROM RESEARCH AND DEVELOPMENT DEPARTMENT, COMMERCIAL SOLVENTS CORPORATION]

The Preparation and Reduction of Nitro Amines Obtained from Aromatic Amines, Formaldehyde, and Nitroparaffins*

BY HAL G. JOHNSON†

Duden, Bock and Reid[1] have shown that 2-nitro-1,3-bisanilinopropane can be prepared by the action of aniline on the reaction product of dimethylamine, formaldehyde and nitromethane. (According to our nomenclature the compound above would be N,N′-diphenyl-2-nitro-1,3-propanediamine.) It is to be noted, however, that the reaction is one of substitution

$$(CH_3)_2NCH_2CH(NO_2)CH_2N(CH_3)_2 + 2C_6H_5NH_2 \longrightarrow (C_6H_5NHCH_2)_2CHNO_2 + 2(CH_3)_2NH$$

Dickey[2] has also shown the preparation of a number of compounds related in structure to those discussed in this paper. His compounds are prepared by the reaction of nitroolefins with amines.

$$HOC_6H_4NH_2 + CH_2{=}CHNO_2 \longrightarrow HOC_6H_4NHCH_2CH_2NO_2$$

All of the compounds prepared by this method must of necessity have at least one hydrogen atom alpha to the nitro group, *i. e.*, it is impossible to prepare a compound having both alpha hydrogen atoms substituted.

Cerf[3] stated that nitroparaffins of the type RCH_2NO_2 react with one mole of N-hydroxymethyldialkylamine. He was not successful in treating two moles, as did Henry,[4,5] Mousset[6] and Senkus,[7] all of whom obtained products of the type

* Prepared for the 1945 Meeting-in-Print of the Division of Organic Chemistry, American Chemical Society.

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(1) Duden, Bock and Reid, *Ber.*, **38**, 2036 (1905).

(2) J. B. Dickey, U. S. Patent 2,292,212, October 5, 1939, "Amino Compound."

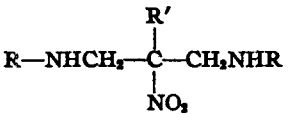
(3) Cerf, *Bull. soc. chim.*, [5] **4**, 1451 (1931); [5] **4**, 1460 (1931).

(4) Henry, *Ber.*, **38**, 2027 (1905).

(5) Henry, *Bull. acad. roy. med. Belgique*, [3] **33**, 412 (1897).

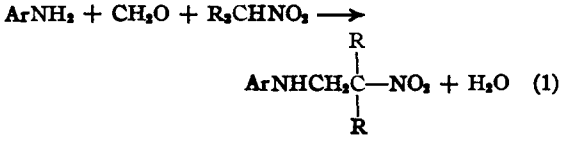
(6) Mousset, *ibid.*, 622 (1901).

(7) M. Senkus, THIS JOURNAL, **68**, 10 (1946).

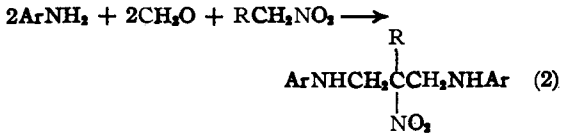


where R is alkyl or aralkyl, and R′ is alkyl or hydrogen. Senkus has discussed in greater detail the limitations of the reaction as studied by Cerf, Henry and Mousset. In view of the successful extension of the reaction by Senkus[7] and Johnson[8] it was a natural outgrowth to include aromatic amines in the study. However, aromatic amines behaved differently. When the reaction was tried in the usual manner there was no evidence of a reaction. It was decided that possibly the low ionization constant of aniline might be a factor. Subsequently this concept proved correct as the reaction did take place in the presence of a strongly basic catalyst. Even then some aromatic amines apparently have such a low ionization constant that the catalyst is ineffective. For example, no reaction could be obtained with diphenylamine or 1- and 2-aminoanthraquinones under any conditions tried and with *p*-nitroaniline it was exceedingly difficult to get a reaction.

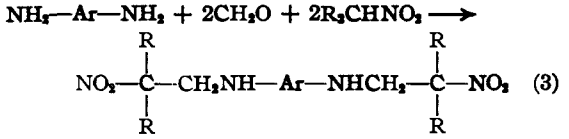
It has been found that aromatic amines can react with formaldehyde and either primary or secondary nitroparaffins to yield products of the following general structure



where Ar represents an aryl group such as phenyl, naphthyl, diphenyl or substituted aryl groups, and R represents an alkyl group



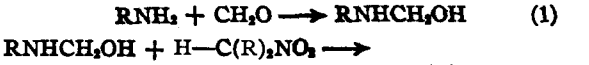
where Ar is an aryl or substituted aryl group, and R is an alkyl group; and diamines give

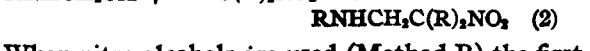


where Ar is aryl and R is alkyl.

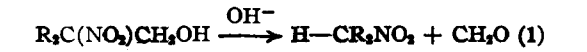
Although the work described herein does not give sufficient evidence for establishing the actual mechanism of the reaction, it is believed that since Method A (the reaction of the amine, formaldehyde and nitroparaffin) is more rapid than Method B (the reaction of the amine with the nitro alcohol), especially with aliphatic amines, the following mechanism is essentially correct

(8) Hal G. Johnson, THIS JOURNAL, 68, 12 (1946).

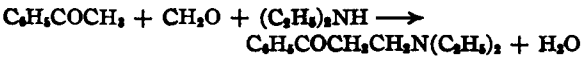




When nitro alcohols are used (Method B) the first step is



followed by the reaction of the amine with the formaldehyde. The formation of nitro hydroxy compounds is a reversible reaction usually carried out in the presence of calcium hydroxide which prevents the reversal of the reaction. Actually the reaction of N-hydroxymethylamines with nitroparaffins is similar to the Mannich reaction where the active hydrogen is furnished by the aldehyde or ketone rather than the nitroparaffin



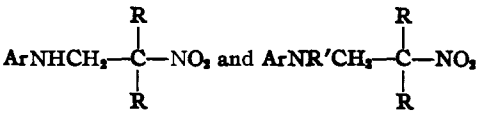
By analogy both α-picoline and quinaldine were tried unsuccessfully as sources of active hydrogen atoms for reaction with N-hydroxymethylamines.

The nitro amines described herein were hydrogenated to the corresponding polyamines and their properties and method of reduction are reported here also.

It is of interest that the color of the nitro derivatives is a function of the substituents present in the aromatic ring. The color of each compound is listed in Table I and it is readily seen that the chloro, sulfonic acid and carboxylic acid derivatives are white whereas the alkyl derivatives are yellow. It was also noted that the position of the group in the ring makes a difference in the color intensity. These phenomena might be explained in the light of Robinson's[9] theory of electronic organic chemistry. The meta position is not conjugated with the ring and does not exert the same electronic effect as do substituents in the ortho and para positions.

Experimental

I. Preparation of Nitro Amines of the Type



Either of two methods can be used for the preparation of aromatic nitro amines illustrated above where Ar represents an aryl group, R represents an alkyl, aryl or hydrogen group (but both may not be hydrogen simultaneously), and R′ represents an alkyl group. These methods are described below using the preparation N-(2-nitroisobutyl)-aniline as an example. Method B was preferred for most of the reactions because it was more convenient to determine the extent of the reaction by the amount of water found.

Method A. Preparation of N-(2-Nitroisobutyl)-aniline from Aniline, Formaldehyde and 2-Nitropropane.—A

(9) Robert Robinson, *J. Soc. Dyers Colorists*, Jubilee Issue, 65 (1934). "Substitution in the Aromatic Nucleus" and "Outline of an Electrochemical Theory of the Course of Organic Reactions," two lectures published by the Institute of Chemistry of Great Britain and Ireland, 30 Russel Square, London W. C. I, 1932.

mixture of one mole of aniline (93 g.), one mole of 2-nitropropane (89 g.), 300 ml. of methanol and 5 ml. of trimethylbenzylammonium hydroxide (40% solution) was placed in a three-necked, one-liter flask fitted with a stirrer, a condenser, and a dropping funnel. The mixture was heated until gently refluxing and the formalin solution (83.4 g. of 35.7% solution which is equivalent to 30 g. on a dry basis) slowly added through the dropping funnel. The addition was made over five to eight hours. The mixture was homogeneous but on cooling to room temperature overnight, crystals of N-(2-nitroisobutyl)-aniline appeared. They were filtered, washed with cold methanol and dried, weight 158.8 g., 80% conversion on the basis of 2-nitropropane; m. p. 64.2°.

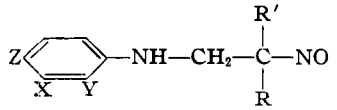
Method B. Preparation of N-(2-Nitroisobutyl)-aniline from 2-Nitro-2-methyl-1-propanol and Aniline.—A mixture consisting of one mole of aniline (93 g.), one mole of 2-nitro-2-methyl-1-propanol (119 g.) and 5 g. of a 40% aqueous solution of tetraethanolammonium hydroxide was placed in a one-liter Erlenmeyer flask and left in a constant temperature bath at 50° for three days. By this time the mixture had solidified and the reaction was complete (roughly judged by pouring off and measuring the volume of water obtained). The solid was filtered off on a Büchner funnel and washed with a mixture of petroleum ether and methanol to remove any traces of unreacted aniline, nitro alcohol and water. The yield of yellow N-(2-nitroisobutyl)-aniline was 181 g.; conversion was 93%, m. p. 63.8°.

Several variations of this later technique have been employed. For example, the nitro alcohol and aromatic amine have been dissolved in benzene and the solution refluxed to remove the water in a Dean and Stark moisture trap. Frequently it has been necessary to freeze the reaction mixture obtained by Method B using Dry Ice because the product was an oily, non-crystalline mass. Alternate freezing and warming would induce crystallization. A number of basic catalysts may be used including sodium hydroxide.

II. Preparation of Nitro Amines of the Type ($ArNHCH_2)_2CRNO_2$.—In this illustration Ar is an aryl group, and R represents an alkyl or aryl group. Actually only Method B described above was used in this synthesis but there is no reason why Method A could not be used. The experiments run with 2-nitro-2-ethyl-1,3-propanediol gave the theoretical amount of water but crystals could not be obtained and the boiling point was too high for purification by distillation. The preparation of N,N'-diphenyl-2-nitro-2-methyl-1,3-propanediamine is described here:

A mixture of 2 moles of aniline (186 g.), 1 mole of 2-nitro-2-methyl-1,3-propanediol (135 g.), and 5 ml. of trimethylbenzylammonium hydroxide was placed in a one-liter Erlenmeyer flask. The nitro diol dissolved in the aniline and the mixture was placed in a constant temperature bath at 50°. A water layer of 36 g. (2 moles) collected at the top after a day and the non-aqueous portion solidified to give yellow crystals. The product was filtered and recrystallized from methanol; yield 201.2 g., 71% conversion; m. p. 110°.

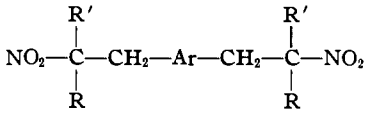
III. Preparation of Compounds of the Type



In the illustration shown X, Y and Z are acidic groups such as COOH and SO_3H but only one is present in each reaction, R is alkyl or hydrogen, and R' can be alkyl, aryl, halogen or hydrogen. Preparations have been made with carboxylic or sulfonic acid groups in the ortho, meta or para positions. Method B was the preferred method for the reasons outlined above. The preparation of N-(2-nitroisobutyl) *p*-aminobenzoic acid is described as an example:

One mole of *p*-aminobenzoic acid (137 g.) was placed in a one-liter flask with 300 ml. of water and one mole of sodium hydroxide (40 g.). As soon as solution was complete one mole (119 g.) of 2-nitro-2-methyl-1-propanol was dissolved in the mixture. The solution was put in a constant temperature bath at 50° for seven days (a shorter time would be sufficient) and then the yellow solution was acidified with dilute (3:1) hydrochloric acid. A pale yellow precipitate was found which was filtered and thoroughly washed with water. A yield of 238.3 g. or 100+% conversion was obtained; m. p. 190°. The material was recrystallized from ethanol.

IV. Preparation of Nitro Amines of the Type



In the structural formula shown R may be alkyl or hydrogen, R' may be alkyl, aryl, halogen or hydrogen, and Ar may be any aryl or substituted aromatic compound such as benzene, biphenyl or naphthalene. The same technique was employed in synthesis as was used in the reaction of aromatic monoamines except that methyl alcohol was used as a solvent and of course two moles of the nitro alcohol were used per mole of diamine. When only one mole of nitro alcohol was tried per mole of diamine the same product resulted. No attempt was made to protect one amino group first and then carry out the reaction.

V. Properties of the Aromatic Nitro Amines.—The nitro amines prepared from aromatic amines are almost without exception solids. The melting points are varied and there seems to be no correlation with structure. These compounds are fairly stable and are usually insoluble in water and have limited solubility in kerosene or saturated hydrocarbons. They are quite soluble in alcohols, benzene, and ketones. The change in color with change in substituents present in the aromatic nucleus is of theoretical interest.

VI. Reduction of Nitro Amines to Polyamines.[10]—The hydrogenation of nitro amines to polyamines was successful on either the crude or pure product. In general, it was found that the conversion to pure polyamine was the same, on the basis of the starting nitroparaffin, whether the nitro amine was crude or pure. The technique used is described in the following general example:

One mole of the pure or crude nitro amine was dissolved or suspended in 300 ml. of methanol and 15 g. of Raney nickel added to the bomb just before reduction. Methanol was used to wash the catalyst into the bomb. The solution was reduced under 1000 lb. sq. in. of hydrogen. Completeness of reduction was determined by the amount and rate of hydrogen absorbed. The mixture from the bomb after reduction was filtered and then distilled through a five-foot packed column containing glass helices. When the original volume of methanol was nearly recovered the distillation was stopped and 300 ml. of benzene added to the flask. Distillation was resumed and the binary mixture of benzene and methanol removed. A Dean and Stark moisture trap was then fitted to the column and the mixture refluxed until no more water collected in the trap. The residue was then distilled first to remove the benzene and finally under reduced pressure. A modified Widmer column was used for the latter distillation.

VII. Properties of the Polyamines.—Most of the polyamines are colorless to pale yellow. A few were low melting solids. All of these amines were only partially soluble in water but completely soluble in benzene, methanol and ether. They all have a mild ammoniacal odor.

Acknowledgment.—The author wishes to thank Dr. J. A. Riddick, Dr. P. C. Markunas and assistants for the analyses reported in this work.

(10) It is pointed out that hydrogenations of nitro amines to polyamines should not be started at high temperatures since a violent reaction may take place because of the exothermic nature of the hydrogenation process.

TABLE I

AROMATIC NITRO AMINES

Starting materials: formaldehyde, 2-nitropropane and amine	Product	Color	M. p., °C.	Conversion, %	Formula	N[f] Content, % Calcd.	Found
	R = 2-Nitroisobutyl						
Aniline[a]	N-(R)-aniline	Yellow	63.8	93	$C_{10}H_{14}N_2O_2$	14.42	14.70
2,4-Dimethylaniline	N-(R)-2,4-dimethylaniline	Yellow	53.8	75	$C_{12}H_{18}N_2O_2$	12.60	12.97
Mesidine	N-(R)-mesidine	Yellow	52.0	41	$C_{13}H_{20}N_2O_2$	11.80	11.85
o-Toluidine	N-(R)-*o*-toluidine	Yellow	50.2	67	$C_{11}H_{16}N_2O_2$	13.45	12.50
m-Toluidine	N-(R)-*m*-toluidine	Pale yellow	64.0	76	$C_{11}H_{16}N_2O_2$	13.45	13.11
p-Toluidine	N-(R)-*p*-toluidine	Yellow	74.5	94	$C_{11}H_{16}N_2O_2$	13.45	13.35
o-Chloroaniline	N-(R)-*o*-chloroaniline	White	53.2–54.5	89	$C_{10}H_{13}ClN_2O_2$	12.26	12.51[d]
m-Chloroaniline	N-(R)-*m*-chloroaniline	Off white	50.2	58	$C_{10}H_{13}ClN_2O_2$	12.26	12.60[d]
p-Chloroaniline	N-(R)-*p*-chloroaniline	White	77.0	80	$C_{10}H_{13}ClN_2O_2$	12.26	12.42[d]
α-Naphthylamine	N-(R)-α-naphthylamine	Yellow	85.0	37	$C_{14}H_{16}N_2O_2$	11.55	11.52
β-Naphthylamine	N-(R)-β-naphthylamine	Yellow	107.3	62	$C_{14}H_{16}N_2O_2$	11.55	11.52
p-Nitroaniline	N-(R)-*p*-nitroaniline	Yellow	136.0	67	$C_{10}H_{13}N_3O_4$	17.57	17.30
Aniline[b]	N-(2-Chloro-2-nitrobutyl)-aniline	Yellow	57.0	56	$C_{10}H_{13}ClN_2O_2$	12.26	12.53[d]
p-Aminodiphenyl	N-(R)-*p*-aminodiphenyl	Yellow	135.1	82	$C_{16}H_{18}N_2O_2$	10.38	10.44
Anthranilic acid	N-(R)-anthranilic acid	White	145.5	89	$C_{11}H_{14}N_2O_4$	11.76	11.61
p-Aminobenzoic acid	N-(R)-*p*-aminobenzoic acid	White	190.0	99	$C_{11}H_{14}B_2O_4$	11.76	11.65
Sulfanilic acid	N-(R)-sulfanilic acid	White	d. 220	89	$C_{10}H_{14}N_2O_5S$	10.21	10.01[e]
N-Methylaniline	N-(R)N-methylaniline	Yellow	38.0	58	$C_{11}H_{16}N_2O_2$	13.45	13.34
Aniline[c]	N,N′-Diphenyl-2-nitro-2-methyl-1,3-propanediamine	Yellow	110.2	71	$C_{16}H_{19}N_3O_2$	14.71	14.52
p-Phenylenediamine	N,N′-Bis-(R)-*p*-phenylenediamine	Yellow	136.0	81	$C_{14}H_{22}N_4O_4$	18.06	18.20
Benzidine	N,N′-Bis-(R)-benzidine	Yellow	166.0		$C_{20}H_{26}N_4O_4$	14.50	14.41

[a] Both methods of preparation, A and B, used here. In all the other reactions Method B was used. [b] With 2-chloro-2-nitro-1-butanol. [c] With nitroethane. [d] Chlorine values also checked in each case. [e] Calcd.: C, 61.80; H, 7.21. Found: C, 61.74; H, 7.22. [f] Dumas nitrogen.

TABLE II

AROMATIC DIAMINES

Product	Conversion %	Formula	Kjeldahl N content, % Calcd.	Found	Boiling point °C.	Mm.	$n^{20}d$	d^{20}_{20}
R′ = (2-aminoisobutyl)								
N-(R′)-aniline	84.4	$C_{10}H_{16}N_2$	17.05	16.94	110	3	1.5575	0.9878
					103	2		
					99	1		
N-(R′)-2,4-dimethylaniline	86.7	$C_{12}H_{20}N_2$	14.57	14.61	128–130	3	1.5319	0.9503
N-(R′)-*o*-toluidine	90.0	$C_{11}H_{18}N_2$	15.72	15.66	104	2	1.5388	0.9662
N-(R′)-*m*-toluidine	98.0	$C_{11}H_{18}N_2$	15.72	15.77	96	1	1.5433	0.9646
					119–120	3		
N-(R′)-*p*-toluidine	85.3	$C_{11}H_{18}N_2$	15.72	15.69	104–107	1	1.5421	0.9680
N-(R′)-*o*-chloroaniline	71.7	$C_{10}H_{15}ClN_2$	14.11	14.19	107	1	1.5527	1.0862
N-(R′)-*m*-chloroaniline	48.4	$C_{10}H_{15}ClN_2$	14.11	14.18	88–89	2	1.5626	1.0943
N-(R′)-*p*-chloroaniline	62.8	$C_{10}H_{15}ClN_2$	14.11	14.27	116–118	0.5	1.5632	M. p. 50
N-(R′)-sulfanilic acid	74.5		11.46	11.43	M. p. d. 300			
N-(R′)-N-methylaniline	69.5	$C_{11}H_{18}N_2$	15.72	15.65	102	2	1.5494	0.9767
N,N′-Diphenyl-2-amino-2-methyl-1,3-propanediamine	81.5	$C_{16}H_{21}N_3$	16.47	16.60	M. p. 52.6		1.0805 d^{35}_{20}	
N-(R′)-*p*-aminobenzoic acid sulfate	82.7	$C_{22}H_{34}N_4O_8S$	10.89	10.94	M. p. 280			
			S, 6.23	6.00				
			C, 51.34	51.96				
			H, 6.62	6.87				

Summary

The reaction of aromatic amines with formaldehyde and primary as well as secondary nitroparaffins has been studied. In contrast to the reactions of alkyl amines the aromatic amines usually require a basic catalyst. Two methods of syntheses have been used: (1) reaction of the amine, formaldehyde and nitroparaffin, and (2) reaction of the amine with the nitroalcohol. The mechanism is thought to be the same in both cases.

Secondary aromatic amines do not exhibit

this reaction unless one of the groups is alkyl. A number of the aromatic nitro amines have been reduced to the corresponding polyamines.

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Physical, Chemical and Immunological Properties of Phosphorylated Crystalline Horse Serum Albumin[1]

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The decisive importance of phosphorylation in synthesis and the transfer of energy in biological processes suggested the introduction of phosphoryl groups into proteins under the mildest possible conditions and a study of the physical, chemical, and immunological changes produced by their introduction. Initial experiments were carried out with crystalline egg albumin,[2] but since it appeared that denaturation accompanied the phosphorylation of this protein, the present study with crystalline horse serum albumin (SA) was undertaken in the hope of avoiding this complication.

Several preparations of phosphorylated serum albumin (PSA) of different P content were made by Rimington's method,[3] as well as by a milder modification in which sodium borate or, preferably, potassium borate was substituted for sodium hydroxide.

Preparation and Chemical and Physical Properties of Phosphorylated Serum Albumin

1. **Preparation of SA.**—Horse serum albumin, crystallized by Adair and Robinson's method[4] and recrystallized five times, was dialyzed against 0.9% sodium chloride solution until free from ammonium sulfate, and then against distilled water until free from chloride ion. After sterilization by filtration through a Chamberland L2 candle, the solution was stored in the refrigerator without preservative. No deterioration was apparent during two years. In contrast to native horse serum, from which the albumin is readily crystallized only when fresh, one purified lot was easily recrystallized after six months of storage. A typical preparation of SA contained 0.05% carbohydrate estimated as galactose by the orcinol method.[5] One-hundred and forty mg. contained 0.18% ash as sodium sulfate, but no phosphorus by the Pregl–Lieb method. The anhydrous protein was obtained by pouring a portion of the aqueous serum albumin solution into 10 vols. of redistilled alcohol and washing the resulting precipitate with alcohol and acetone. After filtration, the precipitate was dried to constant weight at room temperature *in vacuo* over phosphorus pentoxide. Nitrogen was estimated by a modification of the micro-Kjeldahl procedure. The nitrogen content of 15.82% obtained in this way was in good accord with the value, 15.86%, found for another lot which was dried *in vacuo* over phosphorus pentoxide at room temperature directly from the aqueous solution.

2. **Phosphorylation of SA and Isolation of PSA.**—In a typical phosphorylation (PSA3), 1.5 g. of SA dissolved in 55 ml. of water and 55 ml. of 6% $Na_2HPO_4 \cdot 12H_2O$ solution were placed in a 500-ml., three-necked flask immersed in an ice–salt–water bath kept at 0 to −2°. Three grams of freshly distilled phosphorus oxychloride dissolved in 25 ml. of carbon tetrachloride were added dropwise over a period of six hours to the mechanically stirred mixture. Simultaneously, 3 *N* sodium hydroxide solution was added drop by drop to keep the reaction mixture slightly alkaline to phenolphthalein, indicating a *p*H of 8.5 to 9. Since it was feared that the use of strong alkali had caused excessive denaturation in such preparations, solid sodium or potassium borate was substituted in later runs. After addition of the reagents the reaction mixture was placed in the refrigerator until the following day when it was centrifuged in the cold[6] to separate the semi-solid carbon tetrachloride emulsion from the aqueous phase containing the PSA. Lots prepared with sodium hydroxide could be precipitated by addition of *N* hydrochloric acid or preferably acetic acid to about *p*H 3 to 4. After dispersing the centrifuged precipitate in cold water or 0.9% sodium chloride solution the product was dissolved by addition of *N* sodium hydroxide to *p*H 5 to 6 and the isoelectric precipitation was usually repeated. Precipitation was omitted in the case of the less heavily phosphorylated and therefore more soluble lots in the preparation of which potassium or sodium borate had been used. The free phosphate of all lots was reduced to a minimum, usually a few tenths of a μg of phosphorus per ml., by dialysis in the cold against isotonic saline. Traces of phosphate continued to appear in the dialyzate indefinitely, indicating slow spontaneous splitting from the phosphoprotein. The resultant decline in phosphorus content with age could be minimized by storage at *p*H 8 to 9 in the refrigerator or, better, in solid carbon dioxide. The products were analyzed for nitrogen by the micro-Kjeldahl method and for phosphorus after Pregl–Lieb, and the N:P ratios were calculated.

The mode of preparation and purification as well as the properties of all lots of PSA and their fractionation products are described in Table I. Unless otherwise stated, separation and purification of the phosphoprotein was carried out one day after phosphorylation.

3. **Spontaneous Loss of Phosphorus.**—After dialysis to remove inorganic phosphate and analysis for nitrogen and phosphorus, portions of various PSA lots were stored for varying periods and at different temperatures and were then dialyzed and analyzed for phosphorus. In some cases the phosphate which had been split off was determined colorimetrically[7] in the dialyzate.

At 37°, in the presence of toluene, dephosphorylation took place rapidly, especially in slightly acid media. In thirteen days, at *p*H 7.4, PSA4 lost 35 of its 48 phosphoryl groups (*cf.* 4E) (calculated from nitrogen and phosphorus contents on the basis of a molecule of original serum al-

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