[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGICAL CHEMISTRY, HARVARD MEDICAL SCHOOL]

Biochemical and Isolation Studies on a Growth Stimulatory Factor (SSF) for *Staphylococcus Albus* Present in Bovine Plasma

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A purified glycoprotein possessing growth stimulatory activity for *Staphylococcus albus* was obtained from oxalated bovine plasma. The protein was purified 850–1700-fold over the original plasma (nitrogen basis). Electrophoresis and ultracentrifuge data indicate that a highly purified protein preparation was obtained by the isolation procedure. The evidence is presented to suggest that SSF is a biologically active structural peptide present in many different recrystallized proteins. The presence of a chemically similar biologically active protein in urine is also indicated.

Introduction

Previous studies[3,4,5] showed oxalated bovine plasma to be an excellent source of a growth stimulatory factor (SSF) for the bacterium, *Staphylococcus albus*. Studies directed toward the isolation of the biologically active factor contained in bovine plasma resulted in the isolation of a relatively pure glycoprotein which exhibited high activity for the staphylococcus organism. The active compound was concentrated 850–1700-fold (nitrogen basis) from the starting material (oxalated bovine plasma) employing: (1) deproteinization of the oxalated bovine plasma, (2) dialysis, (3) silver precipitation of the non-dialyzable material ("impermeate"), (4) decomposition of the silver proteinate by sodium chloride, and (5) chloroform–amyl alcohol deproteinization, or (6) phenol extraction of the SSF.

Experimental

Isolation of SSF from Bovine Plasma.—The biological activity of the fractions obtained in the isolation procedure was followed by the assay method previously described.[5]

The details of the isolation procedures employed are given in the following paragraphs, the numbers of which correspond to those in the flow chart (Fig. 1). The method has been utilized with equal success for only a few milliliters and for several liters of plasma. The following procedure employs a larger quantity of source material.

(1) **Deproteinization of Oxalated Bovine Plasma.**—Nine and one-tenth liters of plasma was obtained from 19 liters of oxalated bovine blood. The plasma was diluted with an equal volume of water and deproteinated according to the method of Harper, Kinsell and Barton.[6] The diluted plasma was heated to 75–80°, 455 ml. of 5% acetic acid was added slowly with stirring and the heating continued until the temperature reached 80–85°. The mixture was cooled, filtered, adjusted to *p*H 7.0 and assayed. This removal of the readily coagulable and inactive proteins from oxalated plasma resulted in approximately a 40-fold increase in activity with a 100% recovery.

(2) **Dialysis.**—The SSF activity in deproteinated plasma does not dialyze through Visking cellophane membranes. Thus, it was possible to separate the active material from the low molecular weight substances. A plunger type dialysis apparatus designed by Dr. Lawrence Strong[7] was employed for the dialysis at 4° of one liter amounts of deproteinated plasma. The recovery of activity in the impermeate after dialysis was virtually 100% with a corresponding reduction of nitrogen from 0.08 to 0.04 mg. per unit (Fig. 1).

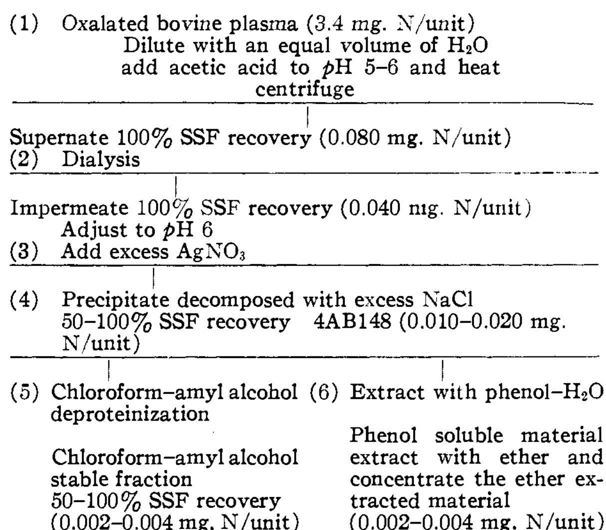

Fig. 1.—Isolation procedure for the fractionation of SSF from bovine plasma. The numbers in parentheses correspond to the numbers in the text.

Dialysis of the deproteinated plasma carried out at different hydrogen ion concentrations (*p*H 2–10) did not change the non-dialyzable character of SSF. Furthermore, the activity of the deproteinated plasma is not rendered dialyzable by autoclaving at 15 pounds for 10 minutes at *p*H 7.5 (Table I).

(3) **Precipitation of SSF in Dialyzed Deproteinated Plasma with Silver.**—The SSF activity in the dialyzed deproteinated plasma was precipitated in the cold with silver nitrate. The impermeate was adjusted to *p*H 6–6.3 and an excess of 10% $AgNO_3$ added (approximately 100 ml. per 2 liters of impermeate). The silver precipitate was collected by centrifugation.

(4) **Decomposition of the Silver Salt of SSF.**—The silver precipitate was suspended in 100 ml. of water and freed of silver by the addition of about 200 ml. of a saturated solution of NaCl, the *p*H being maintained at 7 by the addition of dilute HCl. The silver chloride was removed by centrifugation and washed with 0.9% NaCl solution until the washings were colorless. The combined supernate and washings were dried from the frozen state. This preparation (4AB148) represented approximately a 400-fold purification of SSF from the starting plasma and the nitrogen per growth response unit was reduced from 3.4 mg. to 0.010 mg. (Fig. 1).

(5) **Chloroform–Amyl Alcohol Deproteinization of 4AB-148.**—The fact that certain relatively unstable proteins react with chloroform and amyl alcohol to form a gel[8] afforded a means of precipitating a considerable quantity of non-active proteins. The chloroform–amyl alcohol deproteinization was carried out as follows. To 16 ml. of an aqueous solution (*p*H 6.6) of 4AB148 containing 0.63 mg. N were added 4 ml. of $CHCl_3$ and 1.6 ml. of amyl alcohol. The mixture was shaken for 20 minutes and then centrifuged. The supernate was separated from the $CHCl_3$–protein gel and the gel washed with 5 ml. of H_2O. The su-

(1) United States Public Health Service, National Institutes of Health Predoctoral Research Fellow, 1948–1949. Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

(2) Supported in part by the Higgins Fund.

(3) N. H. Sloane and R. W. McKee, *Federation Proc.*, **8**, 252 (1949).

(4) N. H. Sloane, Thesis, Harvard University, 1950.

(5) N. H. Sloane and R. W. McKee, THIS JOURNAL, **74**, 983 (1952).

(6) H. A. Harper, L. W. Kinsell and H. C. Barton, *Science*, **106**, 319 (1947).

(7) Harvard Medical School, Department of Physical Chemistry; Memoranda and Communications on the Preparation of Normal Human Serum Albumin, 1943.

(8) M. G. Sevag, D. B. Lackman and J. Smolens, *J. Biol. Chem.*, **124**, 425 (1938).

TABLE I

PROPERTIES OF SSF PROTEIN FROM BOVINE PLASMA

Fraction	% SSF activity of original plasma[a]
Dialysis	
Impermeate at *p*H 2, 7 or 10	100
Dialysate at *p*H 2, 7 or 10	0
Impermeate of autoclaved deproteinated plasma (*p*H 7.6)	100
Deproteinization	
Chloroform–isoamyl alcohol deproteinized supernate	0–20
Chloroform–isoamyl alcohol deproteinized supernate reacted with glutathione (2 mg./ml.)	100
Impermeate of chloroform–isoamyl alcohol deproteinized supernate	100
Dialysate of chloroform-isoamyl alcohol stable proteins	Trace
Phenol–H_2O soluble protein	0–20
Phenol-H_2O soluble protein reacted with glutathione	100
Impermeate of phenol–H_2O soluble protein	100
Enzymatic Digestion	
Deproteinated plasma after digestion with:	
(a) Pepsin *p*H 2	100
(b) Trypsin *p*H 8	100
(c) Takadiastase *p*H 4.5	100
Dialysis after Digestion with Pepsin for 3 hours (*p*H 2)[b]	
(a) Dialysate	20
(b) Impermeate	50
Ninhydrin Reaction	
Deproteinated plasma after treatment with ninhydrin	0
Ninhydrin treated deproteinated plasma plus non-treated deproteinated plasma (control)	100[c]
Ninhydrin treated plasma plus glutathione	0
Heat treated deproteinated plasma as in ninhydrin reaction (control)	100

[a] As indicated in the previous paper[5] the error of the assay as carried out in these experiments is about ±10%. [b] Six ml. of digest dialyzed against 12 ml. of H_2O containing 20 mg. of glutathione at 4° for 12 hours. [c] Complete activity of added deproteinated plasma shows absence of toxic products in the ninhydrin treated fraction.

pernate and washing were again treated as before with $CHCl_3$ and amyl alcohol. The combined supernate and wash material were concentrated to dryness *in vacuo* under nitrogen. The dried residue was dissolved in 15 ml. of H_2O and assayed. This treatment resulted in the temporary loss of 80–100% of the SSF activity (Table I) presumably due to oxidation since the activity was restored by treatment with either glutathione or cysteine at *p*H 7.4. This reactivated material was concentrated 850–1700-fold from the plasma (Fig. 1).

The active factor in the purified concentrate was virtually non-dialyzable (Table I). The dialysis was carried out in the presence of glutathione at *p*H 7 and at 4°.

(6) **Phenol Extraction of SSF from 4AB148.**—An alternate procedure to that of chloroform–alcohol deproteinization was the extraction of the active material with phenol. The phenol extraction was carried out in the following manner. Two hundred fifty ml. of a solution containing 166 mg. of protein (4AB148) was extracted 3 times with 25-ml. portions of wet phenol. The combined phenol layers were extracted with 100 ml. of peroxide-free ethyl ether after adding 10 ml. of water. The separated phenol–ether layer was washed 3 times with 20-ml. portions of water. These washings and the phenol–water soluble fraction were further extracted 3 times with 25-ml. volumes of ethyl ether to remove the residual phenol. The phenol-free aqueous phase was heated on a steam-bath to about 60° to remove the residual ether and then dried from the frozen state. This phenol extraction resulted in the temporary loss of 80–90% of the biological activity, which was restored upon treatment of the phenol-soluble fraction with glutathione. This material was concentrated 850–1700-fold from the starting plasma (nitrogen basis). The SSF activity of this concentrate was still non-dialyzable (Table I).

(II) **Physical and Chemical Properties of Purified SSF (Phenol–Water Soluble) Protein.** (1) **Physical Properties.**—The electrophoretic pattern of the phenol-water soluble SSF concentrate of plasma showed a slightly asymmetrical peak in Γ/2 0.1 veronal buffer at *p*H 8.6[9] (Fig. 2). This pattern could be resolved into 3 components. The mobilities of the components and the percentage composition were

%	80	12	8
$-\mu \times 10^5$ (mobility)	6.1	3.6–4.7	7

The ultracentrifuge pattern of this plasma concentrate (*p*H 7.2, Γ/2 0.1 phosphate buffer) showed a diffuse pattern[10] (Fig. 3). This pattern could be resolved into 2 components: (1) an 80% component whose weight average sedimentation constant was 3.4 Svedberg units (*S*) and (2) a 20% component with a sedimentation constant of 5.9 *S*.

(2) **Chemical Composition of Purified SSF.**—The phenol–water soluble SSF material is glycoprotein in nature and contains 3.9% carbohydrate, calculated as an equimolar mixture of mannose and galactose.[11] The phenol–water soluble material contains 0.548% sulfur,[12] and 14.95% nitrogen. The analyses for fourteen amino acids determined by microbiological assays on an acid hydrolysate of the SSF protein are shown in Table II.

TABLE II

AMINO ACID COMPOSITION[a] OF PHENOL-WATER SOLUBLE SSF PROTEIN FROM BOVINE PLASMA

Amino acid	Percentage
Arginine	5.50
Glutamic acid	14.90
Histidine	1.92
Isoleucine	2.15
Leucine	8.60
Lysine	7.20
Methionine	1.02
Phenylalanine	4.27
Proline	3.88
Threonine	4.14
Tyrosine	2.62
Valine	5.05
Aspartic acid	10.10
Glycine	3.23

[a] The amino acid analyses were kindly performed by Dr. Claire Graham of the Wilson Laboratories, Chicago, Illinois.

(III) **Further Chemical Properties of the SSF of Bovine Plasma.**—In addition to the properties of SSF already noted in the previous sections, there are two further important properties: (1) the dialyzability of SSF activity after pepsin digestion of the SSF protein and (2) the inactivation of SSF by ninhydrin.

Dialyzability of SSF Activity After Pepsin Digestion.—The biological activity of the decomposed silver salt (4AB-148) was rendered dialyzable by digestion with crystalline pepsin. The digestion was carried out as follows: an amount of 4AB148 containing 0.378 mg. of N was dissolved

(9) We wish to thank Mr. M. J. E. Budka of the Dept. of Physical Chemistry, Harvard Medical School, for resolving the pattern.

(10) We wish to thank Dr. Eric Ellenbogen, Dept. of Physical Chemistry, Harvard Medical School for resolving the ultracentrifuge pattern.

(11) M. Sørenson and C. C. Haugaard, *Compt. Rend. Trav. Lab. Carlsberg*, **19**, No. 12, 1 (1933).

(12) We wish to thank Dr. Jordi Folch-Pi of the McLean Hospital and Harvard Medical School for the sulfur analysis.

in 9 ml. of 0.01 *N* HCl and digested with 2 mg. of Armour-recrystallized pepsin for 3 hours at 37°. After digestion the *p*H was adjusted to 7, and the material heated on the steam-bath for 10 minutes to inactivate the enzyme. After cooling and centrifuging, the supernate was diluted with an equal volume of water. A 6-ml. quantity was dialyzed at *p*H 7 against 12 ml. of water containing 20 mg. of glutathione. The results are shown in Table I.

Inactivation of SSF by Ninhydrin.—The protein nature of SSF, and its inactivation by ninhydrin, suggests the presence in the molecule of a free amino group alpha to a carboxyl group, or a terminal lysine group.[13,14] The ninhydrin reaction was carried out as follows: seven mg. of ninhydrin was added to 8 ml. of deproteinated plasma. The solution was made alkaline by the addition of 0.1 ml. of 10% ammonium hydroxide, and then heated on the steam-bath for 30 minutes. The solution was cooled and concentrated to dryness *in vacuo*. The dried material was extracted 4 times with 10-ml. portions of hot acetone and then the solvent removed to dryness. The residue was dissolved in 8 ml. of water and then centrifuged. The clear supernate was extracted with 5 ml. of chloroform to remove the residual color, heated to remove the dissolved chloroform, and then assayed. The control deproteinated plasma was treated as above without the addition of ninhydrin. The results are shown in Table I.

(IV) Properties and Preparation of SSF from Human Urine.—As previously indicated[8] there is also present in urine, a material which stimulated the growth of *Staphylococcus albus*. Preliminary experiments dealing with the nature of the factor in human male urine disclosed the following facts indicating the presence of a biologically active material similar to the plasma factor: (a) The SSF activity of urine was non-dialyzable. (b) The activity was destroyed by ninhydrin treatment. (c) Air-oxidation inactivated SSF fractions which were reactivated upon treatment with either cysteine or glutathione. (d) The activity was precipitated by silver at *p*H 6. An SSF concentrate purified 200–400-fold (nitrogen) was prepared from human male urine by dialyzing the urine in the presence of cysteine, precipitating the activity present in the impermeate at *p*H 6 with silver and decomposing the silver precipitate with sodium chloride. The recovery of SSF was virtually 100% and 0.048–0.096 mg. of nitrogen of the concentrate was equivalent in SSF activity to 20.4 mg. of urine nitrogen. (e) The electrophoretic pattern of the decomposed silver concentrate showed an impure preparation with five peaks.

Further study will be required to determine whether or not the plasma and urine factors are identical.

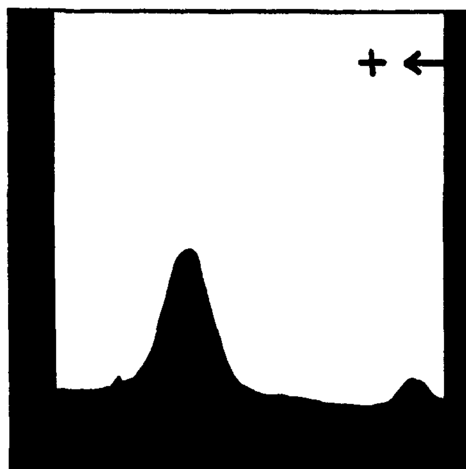

Fig. 2.—Electrophoretic pattern of phenol–water soluble SSF protein, at *p*H 8.6 in Γ/2 0.1 veronal buffer.

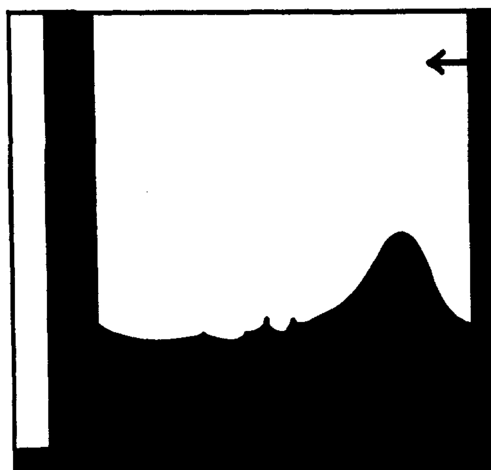

Fig. 3.—Ultracentrifuge analysis of phenol–water soluble SSF protein 40 min. after attaining full speed (Γ/ 20.1 phosphate buffer *p*H 7.2).

Discussion

The proof of the biological effects of macromolecules in microbiological metabolism has been provided by many investigators.[15–18]

In 1945 Sprince and Woolley[19] postulated that the biological activity of strepogenin was due to a portion of the molecular structure of certain proteins. The active peptide moiety apparently contained a precise structural form which was designated as a "structural peptide."

In view of the following considerations, it appears that the biological activity of SSF derived from blood plasma and urine likewise is due to a structural peptide: (a) The activity of SSF protein can be brought into a dialyzable state by peptic digestion. (b) SSF activity is present in recrystallized pepsin-digests of multi-recrystallized proteins (Table III). (c) The biological activity of SSF protein is destroyed upon reaction with ninhydrin.[20] The data strongly suggest the presence in the ac-

TABLE III

RELATIVE CONCENTRATIONS OF SSF ACTIVITY IN MULTI-RECRYSTALLIZED PROTEINS

Pepsin-digested protein	Relative concentration of SSF per mg. nitrogen
Zinc insulin	1.0
Human serum albumin	1.0
Metal combining globulin	1.7
Ribonuclease	2.0
Trypsin	1.3
Hemoglobin	2.5
Phenol-H_2O soluble SSF protein	680.0

(13) S. Ruhemann, *J. Chem. Soc.*, **99**, 792 (1911).

(14) C. Neuberg, *Biochem. Z.*, **56**, 500 (1913).

(15) O. T. Avery, C. M. MacLeod and M. M. McCarty *J. Exp. Med.*, **79**, 137 (1944).

(16) W. H. Price, *J. Gen. Physiol.*, **32**, 301 (1949).

(17) D. W. Woolley, *J. Exp. Med.*, **73**, 487 (1941).

(18) E. Steen, *Acta Path. Microbiol., Scand.*, **XXVI**, 412 (1949).

(19) H. Sprince and D. W. Woolley, THIS JOURNAL, **67**, 1734 (1945).

(20) The inactivation of SSF by ninhydrin does not seem to be attributable to its ability to act as a mild oxidizing agent ["Elseviers Encyclopedia of Organic Chemistry," Vol. 12A, Series III, Elsevier Publishing Co., Inc., New York, N. Y., 1948, page 322] because (1) treatment of the ninhydrin reacted product with glutathione does not restore activity; (2) strong oxidizing agents are necessary to oxidize -SH groups further than -S-S stage [R. Connor, in Gilman, "Organic Chemistry," John Wiley and Sons, Inc., New York, N. Y., 1943, page 851]; (3) it appears that the -SH groups of SSF in both deproteinated plasma and urine are protected from oxidation, since there is no loss of activity after heating at *p*H 10 for 30 minutes.

tive moiety of an amino group alpha to a free carboxyl group,[13] as in the peptide glutathione, or a β-aspartyl peptide or of a terminal lysine group.[14] (d) The reactivation of air-oxidized inactive material by reduction with either cysteine or glutathione is suggestive of the presence of a sulfhydryl group which is necessary for SSF activity. It therefore appears that cysteine is quite probably a constituent of the structural SSF peptide; since the total sulfur content of proteins in most cases can be accounted for by known sulfur-containing amino acids,[21] and it is unlikely that a new sulfur-containing amino acid is present in the SSF protein. Ergothionine which is present in blood has no SSF activity,[5] nor has this compound been shown to be a component of proteins.

(21) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," A.C.S. Monograph 90, page 351, Reinhold Publishing Corp., New York, N. Y., 1943.

The presence of an SSF protein in mammalian blood and the occurrence of biologically active material in natural products suggest that the factor may be important in mammalian metabolism. The role of this protein in cellular metabolism has yet to be determined.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, CARNEGIE INSTITUTE OF TECHNOLOGY]

Studies on the Fischer Indole Synthesis. II[1]

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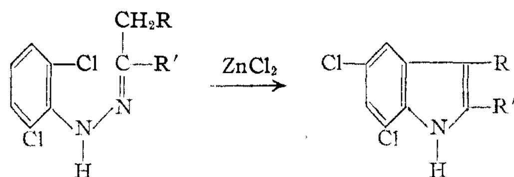
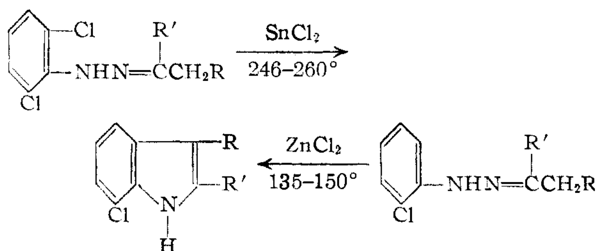
The conversion of five 2,6-dichlorophenylhydrazones to 7-chloroindoles by fusion with stannous chloride, together with the stability of 2-(p-chlorophenyl)-5,7-dichloroindole to the same reagent under the same conditions, indicates that chlorine probably migrates in an electron-deficient or "positive" condition during the conversion of 2,6-dichlorophenylhydrazones to 5,7-dichloroindoles by zinc chloride. Failure to observe any evidence of intermolecular chlorine migration from acetophenone 2,6-dichlorophenylhydrazone to any one of three possible halogen acceptors, in the presence of zinc chloride, lends support to the hypothesis that the chlorine migration is intramolecular. A reaction sequence has been proposed to account for the facts now available about the behavior of 2,6-dichlorophenylhydrazones in the Fischer reaction. This reaction sequence includes a mechanism which postulates a six-ring transition state for the step in which the new carbon-to-carbon bond is formed. The ultraviolet extinction curves for three indoles and a carbazole are reported.

The chlorine migration which occurs during the conversion of 2,6-dichlorophenylhydrazones to 5,7-dichloroindoles[3] requires clarification. The work described in this paper was carried out in order to provide evidence bearing on (a) the nature of the migrating chlorine, and (b) the question of inter- or intramolecular chlorine migration.



The available information is consistent with the postulate that a chlorine atom ortho to the hydrazone linkage rearranges to the para position during the course of the acid-catalyzed transformation of 2,6-dichlorophenylhydrazones to 5,7-dichloroindoles. A large number of analogous halogen migration reactions have been observed to occur when aromatic halides having certain structural features are treated with acidic catalysts,[4] and a considerable body of evidence has accumulated to support the theory that in these cases the halogen atoms migrate in an electron-deficient or "positive" condition. Nicolet and Sampey[5] have shown that halogen atoms so located on aromatic rings as to be susceptible to this type of migration are substituted by hydrogen when the aromatic halides are treated with stannous chloride, but that "non-positive" halogens are not attacked. Accordingly, they have used stannous chloride as a diagnostic reagent to establish the presence of positive halogen. Because stannous chloride is an acidic reagent, it might prove capable of promoting the Fischer reaction. Furthermore, if stannous chloride should catalyze Fischer reactions of 2,6-dichlorophenylhydrazones and if positive chlorine is involved in these reactions, then the stannous chloride should also capture the positive chlorine, and 7-chloroindoles should be formed. Actually, in the present work crystalline 7-chloroindoles have been isolated in 3–17% yields from the forbidding black tars formed when 2,6-dichlorophenylhydrazones of acetophenone, β-acetonaph-



(1) Abstracted from D.Sc. theses by John G. Wallace and E. E. Fisher.

(2) (a) du Pont Pre-doctoral Fellow, 1949-1950; (b) Institute Graduate Fellow in Organic Chemistry, 1947-1948.

(3) (a) R. B. Carlin and E. E. Fisher, THIS JOURNAL, **70**, 3421 (1948); (b) C. S. Barnes, K. H. Pausacker and W. E. Badcock, *J. Chem. Soc.*, 730 (1951).

(4) A bibliography is given by H. Meerwein, P. Hofmann and F. Schill, *J. prakt. Chem.*, **154**, 266 (1940). For a more recent example, see H. T. Huang, D. S. Tarbell and H. R. V. Arnstein, THIS JOURNAL, **70**, 4182 (1948).

(5) B. H. Nicolet and J. R. Sampey, *ibid.*, **49**, 1796 (1927).