was added with stirring during one-half hour a solution of 12.2 g. (0.1 mole) of α-methyl-α-phenylhydrazine and 0.1 mole of the requisite dimethylamide in 25 ml. of dry benzene. Stirring and refluxing were continued for five hours after which the reaction mixture was cooled, treated with 50 g. of ice, and basified with 10 *N* sodium hydroxide. The mixture was extracted with three 100-ml. portions of ether and the combined ether extracts were dried over magnesium sulfate, filtered, and distilled to give the amidrazone.

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Triphenylmethyl Selenocyanate

BY HEINRICH RHEINBOLDT AND HERCULES VIEIRA DE CAMPOS

Triphenylmethyl thiocyanate[1] is, in contrast to the corresponding chloride and bromide, a fairly stable compound. It is not attacked by cold water[2] and so slowly alcoholized by methanol and ethanol that it can be recrystallized without alteration from these solvents[3,4]; it melts without decomposition and can be sublimed[2] and even distilled under reduced pressure.[2] It seemed thus of interest to prepare the hitherto unknown corresponding selenocyanate and to study its behavior.

Triphenylmethyl selenocyanate, $(C_6H_5)_3C\cdot SeCN$, may be prepared by mechanically shaking a solution of freshly prepared triphenylmethyl chloride (8 g.) in pure dry benzene (80 cc.) with a great excess of dry, finely-powdered potassium selenocyanate (8 g.) for five hours at room temperature in a closed brown bottle. Soon after the addition of the potassium selenocyanate, the solution turns slightly yellow and gradually a red pitchy matter separates out together with potassium chloride. After evaporation of the solvent from the filtered benzene solution under reduced pressure in a dry nitrogen atmosphere and protected from daylight, the remaining solid residue appeared as very lustrous yellowish crystals mixed with a few particles of red selenium. This material, after solution in boiling petroleum ether, filtration while hot from the small quantity of gray selenium, and cooling to 0°, yielded colorless and odorless prismatic needles of adamantine luster. The average yield of several preparations was 58%.

Anal. Calcd. for $C_{20}H_{15}NSe$: N, 4.02; Se, 22.67. Found: N, 3.98; Se, 22.70.

The substance, freshly crystallized from petroleum ether, shows the "instantaneous melting point"[5] of 129.5–130.5° and soon after melting decomposes with separation of red selenium. When slowly heated in sealed capillaries it melts at lower and irregular temperatures. Heating to 100° under 3 mm. for many hours does not alter the substance. It decomposes under the action of the sunlight with gradual separation of red selenium, but it stays unaltered for several months when kept in a desiccator carefully protected against the daylight.

The selenocyanate is insoluble in cold and hot water, very slightly soluble in cold petroleum ether, ether, glacial acetic acid, methanol, ethanol and other alcohols; soluble, without decomposition and coloration, in cold dry chloroform, carbon tetrachloride, carbon disulfide, acetone, benzene, aniline, dimethylaniline, pyridine and quinoline (the solutions in the hot basic solvents become yellow without separation of selenium). When kept under a layer of pure alcohols it decomposes slowly at room temperature with gradual separation of selenium and formation of hydrogen cyanide. Heated for a few instants in a water-bath to 60–70° with pure alcohols (methanol, ethanol, propanols, *n*-butanol, isoamyl, benzyl, β-phenylethanol) the selenocyanate dissolves with rapid decomposition which, once initiated, continues, out of the bath, with evolution of hydrogen cyanide and quantitative separation of the selenium. From such solutions the methyl, ethyl and benzyl triphenylmethyl ethers were isolated and identified by their melting points (85, 83 and 105.5°) and mixed melting points with authentic samples of these substances. The alcoholysis of the selenocyanate is therefore much more pronounced than that of the corresponding thiocyanate.[4] In warm aqueous alcohols (for example in 60% methanol or ethanol) hydrolysis takes place instead of alcoholysis with formation of triphenylcarbinol, hydrogen cyanide and selenium. The same happens in aqueous acetone (for example, with 10% water); in a quantitative experiment 99.6% of the selenium content of the selenocyanate was recovered in the elementary state. In this solvent mixture the thiocyanate also is completely hydrolyzed, though in a slower way; thus, from 0.3 g. of the substance, after boiling in a mixture of 25 cc. of 80% acetone for four hours, 96.4% of the theoretical amount of thiocyanic acid formed was found in the solvent. Cold water does not alter appreciably the crystallized selenocyanate, probably because of the insolubility. However, by shaking the finely powdered substance mechanically for twenty-four hours at 20–22° in a closed dark-brown bottle with a great amount of water and some glass beads—a procedure which does not alter the thiocyanate[2]—a slight decomposition of the compound (light reddish color) is observed and the presence of hydrogen cyanide could be proved in the water as well as in the atmosphere inside the bottle. Heated with boiling water, the substance (0.3 g.) presents after one minute a weak reddish color which becomes after half an hour pronouncedly red (m. p. 104–130°) and, nine hours later an examination showed that it had changed into the carbinol (m. p. unsharply at 162°). Aqueous solutions of inorganic bases (6 *N* sodium, potassium or ammonium hydroxide) or mineral acids (3 *N* hydrochloric or sulfuric acid) do not attack the selenocyanate. Concentrated sulfuric acid dissolves them giving initially a yellow color, identical to the halochromic color with the chloride or thiocyanate,[2] which passes slowly to red with turbidity of the liquid. Glacial acetic acid attacks the rather insoluble substance in the cold only very slowly, but at 85–90° dissolves it with a slow separation of selenium; with 80% acetic acid this reaction is much more pronounced, either cold or hot. The action of 98% butyric acid and 85% lactic acid is analogous, but 100% formic acid, which also alters the practically insoluble selenocyanate only very slowly in the cold, brings about at 90° a rapid separation of selenium with an intense yellow color.

Triphenylmethyl thiocyanate and selenocyanate are isomorphous giving a continuous series of mixed crystals.

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RECEIVED SEPTEMBER 20, 1949

(1) K. Elbs, *Ber.*, **17**, 700 (1884).
(2) H. Lecher and K. Simon, *ibid.*, **54**, 635 (1921).
(3) I. Lifschitz, *ibid.*, **58**, 2439 (1925).
(4) A. Hantzsch and A. Burawoy, *ibid.*, **63**, 1181 (1930).
(5) J. Timmermans, "Chemical Species," Chemical Publishing Co., New York, N. Y., 1940, p. 26.

The Precipitation of Insulin by Thiocyanate[1]

BY MARGARET H. SCHWERT AND HANS NEURATH

Preliminary to an electrophoretic investigation of the interaction of insulin with sodium thiocyanate, Volkin[2] found that, whereas thiocyanate in-

(1) Part of a thesis submitted by Margaret H. Schwert to the Faculty of the Graduate School of the University of Minnesota, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.
(2) Volkin, *J. Biol. Chem.*, **175**, 675 (1948).

creased the solubility of insulin in the isoelectric region, below *p*H 5 this anion decreased the solubility of the protein. At *p*H 2, where insulin is readily soluble in the presence of 0.15 *N* NaCl, the addition of the same concentration of thiocyanate resulted in practically complete precipitation of the protein. These observations, together with Volkin's[2] findings of a binding of thiocyanate by insulin at *p*H 5.8, have prompted the present experiments on the precipitation of insulin by potassium thiocyanate. In this analysis, the effects of salt and protein concentrations, and of *p*H and temperature on precipitation have been correlated with the influence of the same external factors on the equilibrium of the 12,000 molecular weight unit of this protein with its higher (tri- or tetra-) polymeric forms.[3,4]

Experimental

Crystalline Zn insulin (Eli Lilly and Company, Lot 987267) was suspended in water and dissolved by the addition of 0.1 *N* nitric acid. The *p*H was then adjusted to the desired value by the addition of more acid or of 0.1 *N* NaOH. The highest *p*H at which a stable, 1% insulin solution could be obtained was *p*H 3.95. A 10% stock solution of potassium thiocyanate (C. P.) was made up in distilled water and diluted as required, with the simultaneous addition of 0.1 *N* nitric acid to adjust the *p*H to the desired value. While at *p*H 1.6 and 2.5, the insulin solutions were sufficiently self-buffered to resist any change in *p*H on the addition of an equal volume of the isohydrionic potassium thiocyanate solution, at *p*H 3.0 and 3.95, the addition of the salt solution caused a marked increase in *p*H (0.6 *p*H unit at *p*H 3.95). Hence, in experiments at these latter *p*H values, the *p*H of the salt solution was adjusted so as to compensate for this incipient *p*H rise. Precipitation was carried out by adding to 1 ml. of the potassium thiocyanate solution, in a heavy walled ignition tube of about 15 ml. capacity (16 × 125 mm., o. d.), 1 ml. of the insulin solution. The tubes were stoppered with serum caps and equilibrated for twenty-four hours at the desired temperature. In experiments at 3.3°, equilibration was accomplished by fitting the tubes radially on a rotating wheel which was submerged in a constant temperature bath. Equilibration at 25° was done simply by attaching the tubes to a rack which was connected with the shaker of a Warburg bath (American Instrument Company). After twenty-four hours, the tubes containing the solutions were centrifuged at 3,500 r. p. m. for approximately twenty minutes, at 1° and 25°, respectively. The supernatant solution was drawn off with a pipet and an aliquot diluted for analysis of protein concentration, in a Beckman D. U. photoelectric quartz spectrophotometer. The unknown protein concentration was determined by interpolation from a linear plot of the optical density at 276 mμ against protein concentration, the latter having been previously established by Kjeldahl N analysis (16.6% protein N).[5] It was found that up to about 0.1% insulin could be satisfactorily determined at this wave length. A solution of 0.2% potassium thiocyanate in nitric acid, at *p*H 2.5, gave an optical density of about 0.007 as compared to 0.563 for a solution containing 0.06% insulin.

Results

The dependence of the degree of precipitation, expressed as per cent. of the total protein, on the concentration of potassium thiocyanate, given herein in g. per 100 ml. of solution, is shown in Fig. 1 for three different initial insulin concentrations. These results show that the higher the initial protein concentration, the less potassium thiocyanate is required to produce the same fractional degree of precipitation. Precipitation experiments carried out at two different temperatures under otherwise identical conditions reveal that insulin is less soluble in thiocyanate at the lower temperature (Fig. 2). When at constant initial insulin concentration and temperature, the *p*H is varied, results such as those given in Fig. 3 are obtained. It is apparent that, within the *p*H range studied, the solubility of insulin in thiocyanate decreases with increasing *p*H. Thiocyanate-precipitated insulin, after removal of the salt by dialysis against water and nitric acid (*p*H 2.45), retained undiminished biological activity (24 units/mg.).[6]

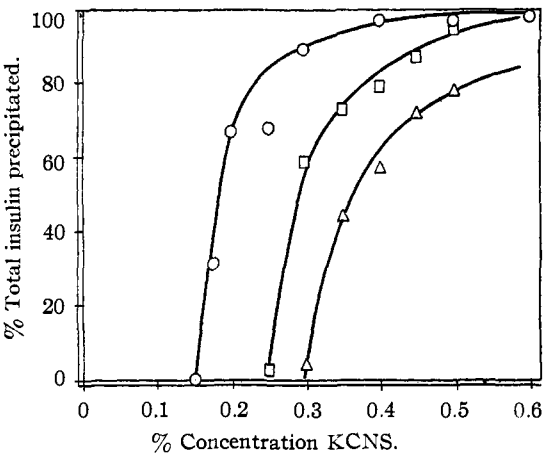

Fig. 1.—Influence of insulin concentration on the precipitating action of KCNS at *p*H 2.5, 25°: ○, 1% insulin; □, 0.5% insulin; △, 0.25% insulin.

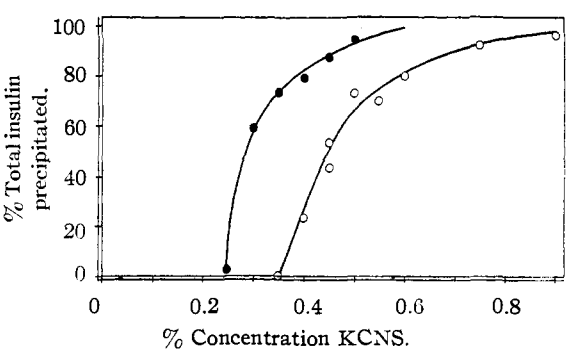

Fig. 2.—Influence of temperature on the precipitating action of KCNS on 0.5% insulin at *p*H 2.5: ●, 3.3°; ○, 25°.

At constant *p*H and temperature, the present

(3) Gutfreund, *Biochem. J.*, **42**, 156, 544 (1948).

(4) Ellenbogen, Thesis, Harvard University, 1949; private communication from Dr. J. L. Oncley.

(5) This value for the nitrogen content pertains to this particular preparation of insulin and was determined by the Lilly Research Laboratories, Eli Lilly and Company.

(6) We are indebted to Dr. E. D. Campbell of the Lilly Research Laboratories for these and other biological assays.

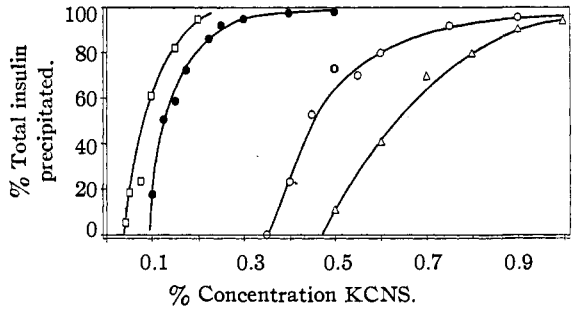

Fig. 3.—Influence of pH on the precipitating action of KCNS on 0.5% insulin at 25°: △, pH 1.6; ○, pH 2.5; ●, pH 3.5; □, pH 3.9.

experimental results can be expressed by the salting-out equation[7]

$$\log S = \beta' - K_s'(\Gamma/2)$$

where S is the solubility of the protein in g. per liter and $\Gamma/2$ the ionic strength. However, unlike typical salting-out phenomena, the determined values of K_s' were not independent of pH and temperature but decreased as either of these factors was decreased.

The values of β' showed a gradual increase as the pH decreased. From the variations of K_s' it was concluded that factors other than those which govern the salting-out of proteins by concentrated salt solutions partake in the present precipitation reaction. The most important of these is specific combination between protein and thiocyanate, as evidenced by the following findings: (1) the shift in pH as revealed by the addition of thiocyanate to unbuffered insulin solutions at pH 3.0 or higher; (2) the shift in electrophoretic mobility at pH 5.8, observed by Volkin,[2] and (3) the failure of other univalent anions, such as chloride, to cause the precipitation of insulin in equal or 5 times higher salt concentrations. Another reason for the failure of the salting-out equation to apply strictly to the present system is the change in the degree of association of the insulin monomer with changing pH, and ionic strength.[4]

The present findings are in full agreement with the assumption that the precipitating action of thiocyanate is directed primarily toward the tri- or tetrameric form, I_3 or I_4 (considering I as the 12,000 molecular weight unit),[4] and that any factor which shifts the molecular equilibrium toward the aggregated state likewise promotes precipitation by thiocyanate. These factors are:[3,4] (1) increase in protein concentration, (2) increase in pH above pH 2, (3) decrease in temperature and (4) increase in ionic strength. The results of other types of measurements on the effect of thiocyanate on insulin[8] are in agreement with this view.

(7) Cohn and Edsall, "Proteins, Amino Acids and Peptides," New York, N. Y., 1943.

(8) Frederieq and Neurath, THIS JOURNAL, 72, 2684 (1950).

Acknowledgment.—Our thanks are due to the Lilly Research Laboratories, Eli Lilly and Company, for the supply of crystalline Zn insulin and for a research grant which have made this work possible.

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RECEIVED SEPTEMBER 19, 1949

Preparation of 2'-Nitro-4'-methoxy-5-chlorodiphenylamine-2-carboxylic Acid

BY DAVID SHAPIRO

In contradistinction with the claim of Knunyants and Benevolenskaya,[1] the preparation of the above acid from 2,4-dichlorobenzoic acid and 3-nitro-4-aminoanisole gave only poor yields, and the synthesis from 4-chloroanthranilic acid and 3-nitro-4-bromoanisole,[2] suffers from the difficult accessibility of the starting material. The acid can be prepared conveniently by nitration of 4'-methoxy-5-chlorodiphenylamine-2-carboxylic acid which is a commercial intermediate in the Atabrine synthesis.

The solution of 69.5 g. of 4'-methoxy-5-chlorodiphenylamine-2-carboxylic acid in 550 cc. of glacial acetic acid, was cooled with stirring to 6° and slowly treated with a mixture of 19 cc. of nitric acid (sp. gr. 1.402) and 50 cc. of glacial acetic acid. The temperature was slowly raised to 50° and kept at this level, until the mixture became brick colored. Cold water was added and the red crystals were collected and washed with water (60 g., 75%). The acid crystallizes from 40 parts of butanol, melts at 270–272°, and shows no depression of the m. p. with a sample prepared according to the Russian authors.[1]

Anal. Calcd. for $C_{14}H_{11}ClN_2O_5$: N, 8.7. Found: N, 8.7.

(1) Knunyants and Benevolenskaya, *J. Gen. Chem.* (*U. S. S. R.*), **10**, 1415 (1940) (*C.A.*, **35**, 3642 (1941)).

(2) Samant, *Ber.*, **75**, 1008 (1942).

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RECEIVED NOVEMBER 4, 1949

An Improved Procedure for the Condensation of Potassium Phthalimide with Organic Halides

BY JOHN C. SHEEHAN AND WILLIAM A. BOLHOFER[1]

In the usual method of conducting the Gabriel condensation, potassium phthalimide and the organic halide are heated together without solvent or in the presence of a non-polar, high-boiling solvent (such as xylene). The insolubility of potassium phthalimide under these conditions hinders the reaction, necessitating prolonged heating (two to twenty-four hours) at relatively high temperatures (100–150°). This results in lowered yields and impure products.

We have found that by carrying out the condensations in dimethylformamide, in which potassium phthalimide is appreciably soluble, a mild exother-

(1) Swift Amino Acid Fellow, 1947–1949.