(1 mole) of benzylamine was treated with 40.5 g. (0.5 mole) of ethylene chlorohydrin at 100° for eight hours. The reaction mixture was cooled, treated with excess 50% sodium hydroxide solution, and the amine layer distilled. N-Benzylmorpholine, b. p. 101–103° (1.5 mm.),[23] $n^{25}D$ 1.5131.

Anal. Calcd. for $C_{11}H_{15}NO$: N, 7.9. Found: N, 7.9.

N-Benzyl-N-(2-thenyl)-2-aminoethanol (XII).—2-Thenyl chloride, (39 g., 0.63 mole) was added to 95 g. (0.63 mole) of 2-benzylaminoethanol,[24] keeping the temperature below 100–105°. The mixture was held at 100–105° for six hours, cooled, treated with water and 58 cc. of 50% sodium hydroxide solution, and the non-aqueous layer separated and dried over anhydrous sodium sulfate. On fractionation, XII was obtained in 40% conversion and 79% yield.

(23) Gabriel and Stelzner, *Ber.*, **29**, 2386 (1896), reported b. p. 260–261°.

(24) Rumpf and Kwass, *Bull. soc. chim.*, **10**, 347 (1943).

N-Benzyl-N-(2-thenyl)-2-chloroethylamine Hydrochloride (XIII).—To 40 g. (0.16 mole) of XII in 100 cc. of chloroform there was added a solution of 26 g. (0.22 mole) of thionyl chloride in 75 cc. chloroform, keeping the temperature of the reaction mixture at 35–40°. After one hour at 35–40°, the mixture was heated at reflux (64°) for one hour, cooled, and the solid product filtered off and washed with chloroform. The crystalline XIII was recrystallized from a mixture of benzene and chloroform with the aid of decolorizing charcoal; yield, 68%.

Summary

A number of new compounds related to known products of high antihistaminic activity have been described. N,N-Dimethyl-N′-(5-chloro-2-thenyl)-N′-phenylethylenediamine hydrochloride was found to be the most active of the new compounds prepared.

ST. LOUIS 4, MISSOURI RECEIVED AUGUST 1, 1949

[CONTRIBUTION FROM THE DEPARTEMNT OF CHEMISTRY, COLLEGE OF MEDICINE, NEW YORK UNIVERSITY]

Determination of Organic Compounds as Isotopic Derivatives.[1] II. Amino Acids by Paper Chomatographic and Indicator Techniques[2]

BY ALBERT S. KESTON, SIDNEY UDENFRIEND[3] AND MILTON LEVY

In the isotopic derivative method of analysis,[4] as applied to amino acids, the products of protein hydrolysis are quantitatively converted to *p*-iodobenzenesulfonyl derivatives (pipsyl derivatives) with a reagent labeled with I-131. The radioactive pipsyl derivatives are separated and purified using non-isotopic pipsyl derivatives as carriers in sufficient quantity to allow effective purification by crystallizations, distribution between solvents and selective adsorptions. The analytical result is calculated from the specific activity of a pure sample of the carrier isolated from the mixture and does not depend on complete recovery. The considerable losses accepted in the attainment of purity do not affect the quantitative nature of the result.

Considering the amount of materials recovered in a pure form, paper chromatography[5] seems a more efficient method of separating and purifying substances than is recrystallization. It could not be applied to the rather large amounts of material present after the addition of carriers as used in the original isotopic derivative technique.[4] We tried some experiments on the radioactive pipsyl derivatives on paper chromatograms without addition of carriers. All steps were carried out in a standard quantitative way, avoiding all losses.[6] The inconveniences of this standard type of operation, however, led us to the use of pipsyl derivatives labeled with S-35 as "indicators."[6] The added material is called an indicator since its function is to indicate the fraction of unknown recovered and not to provide sufficient material for the required operations as carriers ordinarily do. After the indicator has been added and mixed with the unknown further manipulations need not be quantitative. The essential feature of their use is that the fractional recovery of the indicator at any stage is also the fractional recovery of the compound of interest. No operation capable of significantly separating the two types of isotopic compounds is used. The amount of indicator added initially must of course be known in counts of S-35 for the sulfur labeled indicators or in absolute quantities if the molar radioactivity of the S-35 is known.

The radiation characteristics of I-131 and S-35 are different and it is easy to determine the proportion of the total registered ionizing events in a mixture due to each by the use of aluminum foil filters. The analysis is calculated from the ratio between S-35 counts (y) and I-131 counts (x) in a pure sample of the compound isolated. If T_s is the number of counts added with the indicator (S-35 counts) and C_r is the molar activity (counts per mole) of the I-131 reagent used, there are in the unknown mixture $u = xT_s/yC_r$ moles of compound estimated. Constancy of the ratio x/y in successive portions of a chromatographic band establishes the purity of the derivative in it and fail-

(1) This work was done under a grant from the American Cancer Society to Dr. R. K. Cannan, made on recommendation of the Committee on Growth of the National Research Council.

(2) A preliminary report of some of this material was presented at the meeting of the American Society of Biological Chemists: *Federation Proceedings*, **8**, 213 (1949).

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(4) A. S. Keston, S. Udenfriend and R. K. Cannan, (a) THIS JOURNAL, **68**, 1390 (1946); (b) **71**, 249 (1949).

(5) E. Comsden, A. H. Gordon and A. J. Martin, *Biochem. J.*, **38**, 224 (1938).

(6) A. S. Keston, S. Udenfriend and M. Levy, THIS JOURNAL, **69**, 3151 (1947).

ure to attain constancy in successive portions of the band demonstrates its impurity.

When indicators have been added, chromatography can be considerably simplified by using preliminary counter-current extractions to isolate "groups" of pipsyl derivatives for subsequent chromatography. By decreasing the number of compounds to be separated on the strips greater certainty of identification by retardation factors is achieved. Convenient groupings are suggested by a consideration of the distribution coefficients in Table I. In the procedure described in the present communication the five most hydrophilic amino acids (glutamic acid, aspartic acid, hydroxyproline, serine and threonine) are treated as a single group, called group I hereafter.

TABLE I

DISTRIBUTION OF PIPSYLAMINO ACIDS: ENTERED IS K = CONCENTRATION IN ORGANIC PHASE/CONCENTRATION IN 0.2 N HCl IN WATER[a]

Organic phase	CCl_4	$CHCl_3$	$(CH_2Cl)_2$	$(C_2H_5)_2O$	$C_2H_5O_2$-CCH_3
Pipsyl acid	0	0	0.003	0.015	0.11
Pipsylaspartic acid	0	.01	.05	15	90
Pipsylglutamic acid	0	.01	.07	17	100
Pipsylserine	0	.05	.15	4.2	22
Pipsylthreonine	..	.16	.41	8.0	45
Pipsylhydroxyproline	0.01	.19	.56	4.7	50
Pipsylglycine	.01	.63	2.1	44	100
Pipsylalanine	.04	3.0	5.5	100	100
Pipsylmethionine	.26	24	20	.....	.....
Pipsylvaline	.48	24	40	100	100
Pipsylproline	.66	50	50	.....	.....
Pipsylphenylalanine	.75	50	100	.....	.....
Pipsylleucine	1.24	100	100	.....	.....
Pipsylisoleucine	1.60	100	100	100	100

[a] 0 means less than 1% in the organic phase and 100 means more than 99% in the organic phase.

In the separation of group I some radioactive pipsylglycine remains with it. This is removed by a "wash out" procedure by precipitating added inactive pipsylglycine from the mixture.

The developing systems used to separate the derivatives on paper are selected so as to give retardation factors near 0.5. Thus a mixture of butanol and isopropyl alcohol is used when the derivatives of aspartic and glutamic acids are to be separated giving chromatogram Ia. To separate the three hydroxy acids butanol gives better results. The chromatogram of group I so developed is called Ib. The derivatives are applied to the paper as ammonium salts and the solvents are equilibrated with 1 N ammonia in water before use. Under these conditions the higher alcohols give lower retardation factors. Some of these will be useful with group II and III derivatives.

Radioautographs prepared from the chromatograms are very useful in exactly locating the bands and delineating them. The autographs are used as a guide in cutting the bands and in dividing them into strips for demonstration of isotope ratios.

The following procedure is one which has been specifically developed and used for the five amino acids of group I. Many variations are possible because of the versatility of the indicator procedure. We deem it advisable to give the procedure in some detail because it is illustrative of the principles rather than because it is necessarily the most efficient process. The procedure has been divided into steps which will be outlined first, then given in detail.

Outline of Procedure

1. The amino acids are converted to I-131 pipsylamino acids by reaction with I-131 pipsyl chloride.

2. The five indicators, S-35 labeled derivatives (see below for preparation) of aspartic acid, glutamic acid, serine, threonine and hydroxyproline, are accurately measured and thoroughly mixed with the product of step 1.

3. The *p*-iodobenzenesulfonic acid arising from the excess of reagent used in step 1 is removed by a counter-current ether–acid distribution.

4. I-131 pipsylglycine is removed by repeated precipitation of inactive pipsylglycine from a solution of the products of step 3.

5. A chloroform–acid distribution is used to remove most of the residual pipsylglycine and all of the pipsylalanine and other more chloroform soluble derivatives containing I-131.

6. The pipsyl derivatives in the aqueous layer are brought into a small volume and applied to paper strips. For aspartic acid and glutamic acid these are developed with isopropyl alcohol–butanol–ammonia (Group Ia). For the others, (Group Ib) butanol–ammonia is more satisfactory.

7. Radioautograms of the developed strips are prepared.

8. Guided by the radioautograms the appropriate bands are cut out and divided into several (usually 5 or more nearly equal) strips. The strips are separately eluted and the eluates mounted on planchettes.

9. The radioactivities of the deposits are counted with ($\bar{c}$ counts) and without ($\bar{s}$ counts) a suitable aluminum filter. A constant ratio in successive strips demonstrates purity. Suitable decay standards are also counted.

10. The results are calculated from the average $\bar{c}/\bar{s}$ values and the other appropriate data as shown in section 10 below.

Detailed Procedure

1. Neutralized protein hydrolysate of 0.2–1.0 mg. of protein is treated with I-131 pipsyl chloride exactly as described previously for quantitative conversion to I-131 pipsylamino acids. The preparation of the reagent itself is described in a previous publication[4b] and is used at the same level of activity.[7]

(7) The carrier free I-131 now supplied by the Atomic Energy Commission contains considerable amounts of sulfite. Sulfite inhibits the replacement of the diazo group of diazobenzenesulfonic acid by iodide. We overcame this by bringing the solution supplied to dryness after adding a milligram of potassium iodide and dissolving the residue in 0.2–0.3 ml. of 6 N sulfuric acid per 100 mc. of I-131. The sulfur dioxide is removed sufficiently by volatilization to give yields of 60–80% whereas when sulfite was not removed yields of 10–30% were obtained. The acid is neutralized before salting out the sodium pipsylate.

2. To the mixture resulting from 1, S-35 pipsyl derivatives of glutamic acid, aspartic acid, serine, threonine and hydroxyproline are added in accurately measured amounts, and thoroughly mixed. About 0.3–0.9 micromol of each is added. The indicators are stored as ammonium salts in aqueous solution in the cold room. Their preparation as well as that of S-35 pipsyl chloride is described below. The concentrations of the S-35 pipsylamino acids solutions are determined by use of the absorption at 250 mμ measured by a Beckman D. U. spectrophotometer as described previously.[4b] The following millimolar extinction coefficients are used: pipsylglutamic acid 15.5, pipsylaspartic acid 15.5, pipsylserine 15.8, pipsylthreonine 15.8, pipsylhydroxyproline 16.5.

3. The mixture of indicators and unknowns is transferred to a 60-ml. separatory funnel, acidified to about 0.2 *M* hydrochloric acid and diluted to 15 ml. with 0.2 *M* acid. An equal volume of ether (saturated with water) is added and the two phases are shaken together. After separation the aqueous phase is removed to separatory funnel 2 containing 15 ml. of ether and a fresh 15 ml. of 0.2 *M* hydrochloric acid (saturated with ether) added to funnel 1. The two funnels are shaken and the aqueous layer of 2 transferred to 3 containing 15 ml. of ether, the aqueous 1 transferred to 2 and fresh ether saturated aqueous phase added to 1. This cycle is continued until four separatory funnels are in use. Thereafter no further aqueous layer is added to 1 but the process is continued until all aqueous layers have passed out through funnel 4. This process removes 99.99% of the pipsyl acid but leaves practically all of even the most water-soluble derivatives, pipsylserine, in the ether. It requires about fifteen minutes. The ether layers are combined, the ether evaporated, the residue dissolved in 1 ml. of dilute sodium hydroxide and transferred to a test-tube.

4. Fifty mg. of normal pipsylglycine is dissolved with just enough sodium hydroxide to make 1 ml. of solution in water. Sixty cmm. of this solution is added to the solution resulting from step 3, thoroughly mixed in, and a drop of concentrated hydrochloric acid is added. The tube is warmed to dissolve the precipitated pipsylglycine and is then permitted to cool slowly. The solubility of pipsylglycine is 0.3 mg. per ml. at room temperature so that 90% of the pipsylglycine precipitates, carrying down the same fraction of its isotopic analog. There is a certain amount of coprecipitation of the other derivatives as well. After equilibrium has been attained at room temperature the tube is centrifuged and the supernatant removed to another tube. Three mg. of the same pipsylglycine as before is added, the warming and cooling repeated and the second washout again removes 90% of the radioactive pipsylglycine. In general the 1% of I-131 pipsylglycine remaining may be neglected but in some proteins (silk and gelatin) the large amounts of it present may require a third and even fourth washout.[8]

5. The acid supernatant solution is removed to a small separatory funnel and diluted to 15 ml. It is extracted four times with equal volumes of chloroform. The chloroform is discarded and the aqueous phase is extracted with an equal volume of ether. The aqueous phase is discarded.

6. The ether layer is evaporated to dryness in a 50-ml. conical centrifuge tube and the residue taken up in about 0.2 ml. of 3 *M* ammonia in 50% (by volume) alcohol. The solvent is used to wash down the sides of the tube. By means of a suitable capillary pipet 1.5–15 cmm. of the ammoniacal alcoholic solution is applied as a transverse line 2 cm. long at a point about 10 cm. from one end of a strip of Whatman no. 1 filter paper 3 × 57 cm. The place is marked with a pencil. At least two such strips are prepared. The solvents and excess ammonia are dried out of the paper by the use of an infrared lamp.

The apparatus used in developing the chromatograms may be varied considerably. We have generally used a petri dish mounted on a stand and carrying an octagonal glass frame over which the strips are draped. The whole is covered by a bell jar during the development. The solvents used are shaken with an equal volume of 1 *M* ammonia before use. The floor of the container has a petri dish for the aqueous layer, while the upper petri dish contains the solvent layer; the walls of the bell jar are wetted with both phases. For the chromatograms to be used for glutamic and aspartic acid estimations a mixture of 35 volumes of isopropyl alcohol and 50 volumes of normal butanol is the developing fluid. For hydroxyproline, serine and threonine, normal butanol is suitable. After the solvents have run about 25 cm. past the initial spot (in 16–24 hours) the strips are removed from the bell jar and allowed to dry.

7. Location spots are placed on the strips with radioactive ink. This is ordinary ink to which a small amount of I-131 has been added. A spot is placed over each of the pencil marks at each side of the original sample position and at each side of the paper as far down the strip as the film size will allow. These spots enable one to orient the strips with respect to the radioautograph made from them. The prepared strips are mounted with Scotch Tape on the separation sheets used in packaging X-ray film. It is convenient to number the strips with radioactive ink to identify the radioautograms. Type K industrial X-ray film (Eastman Kodak Co.) generally gives a useful autograph in one to four hours depending on the age of the I-131 and S-35 preparations and is used when rapid results are desired. For weekend or overnight exposures the slower Eastman Blue Brand X-ray film is used. The exposure times may be varied greatly since radioautography is used only to locate the bands of interest.

8. The chromatograms are matched to the developed films by means of the radioactive ink spots and their traces on the film. The outlines of the bands are traced in pencil on the paper using a ground glass plate and lamp for viewing. In general the bands are irregular at their edges but clearly separated. Typical radioautographs are shown in Fig. 1.

Each band is cut crosswise into five or more successive strips (2–5 mm. wide) and each strip placed in a 10 × 75 mm. test-tube containing 0.1–0.2 ml. of 0.1 *N* ammonia. After sufficient soaking to elute most of the pipsyl compounds, nearly all of the water is removed by using a fine tipped micropipet and placed as a drop near the center of a planchette.[9] The water is evaporated under an infrared lamp, leaving the solids in a circle about 3–5 mm. in diameter. The samples are nearly weightless and corrections for self absorption are insignificant. Planchette holders consisting of $^7/_8$ inch o. d. iron washers stuck on masonite with "Duco" cement aid in the management of the planchettes after they are prepared.

9. The counting equipment consists of a Technical Associates Scaling Unit and an end window (mica 6 mg./sq. cm.), tetramethyllead filled counting tube (Technical Associates TA-SB-1) mounted in a lead shield (Technical Associates Model LS-2). Arrangements are made to place an aluminum foil filter over the sample without disturbing the geometry. The aluminum foils are mounted on brass or aluminum frames which rest on the turned up edges of the can covers and hold the foils just above the samples. The aluminum foils used vary from 2.0 to 3.5 mils. in thickness. The activities of the sample without filter in counts per minute ($\bar{s}$) and with filter in counts per minute ($\bar{c}$) are read using sufficient time to make the probable error in each case 1% or less. Counts are of course corrected for background, which averages 20 counts per minute. Appropriate standards are counted just before and just after each set of band strips. The average ratio (r) of the counts with to those without the filter, for consec-

(8) Losses by coprecipitation may become so great that it is worth while to change to a more extensive counter-current distribution to remove pipsyl glycine.

(9) We use "C and E" can covers, $1^1/_8$" in diameter, and made of tin plate. These were obtained from the American Can Co., and have a small rim which is very useful for handling the planchettes with forceps or with an otological snare. The counting shields are equipped with appropriate slides and positioning devices which accommodate these covers.

utive strips giving the same value within the error of measurement, is used for the calculation. If the consecutive strips show differences in r the band is not pure. Sometimes rechromatographing the material on the planchettes will give a pure band from which the analysis may then be made.

Standards.—Several systems of calibration in terms of the reagents used have suggested themselves and have been used. We believe the following to be most reliable in that wide variations in placement of the sample has little measurable effect on the result. Two samples of pipsylalanine are prepared. One is made from the I-131 reagent available and the other from the S-35 reagent in use. Each is carefully purified, dissolved in a small amount of ammoniacal water and its concentration estimated by measuring the absorption at 250 mμ of a suitable dilution in 0.2 N hydrochloric acid. The molar extinction coefficient of pipsylalanine is 15,800.[4b] A standard solution is prepared by mixing carefully measured volumes of the two solutions so that a moles of I-131 pipsylalanine and b moles of S-35 pipsylalanine are present in the volumes of the so ution used in preparing the standard planchette. A suitable portion of this mixture is mounted on a planchette as described above for the unknown. This standard is counted with and without filter and the ratio of counts, r_d, is used in the calculations. We have counted such standards with the deposit directly under the center of our G-M tube and with the deposit very near the outer edge of the planchette. While the absolute counts recorded differed by more than 20%, r_d was unchanged within 1%.

10. Calculation.—The data and equations used in the calculation of the amount of unknown amino acid in the original sample follow. The symbols are

- $\bar{c}$ = counts obtained from a planchette with the aluminum filter in place
- $\bar{s}$ = counts obtained from the same planchette without filter
- r_i = $\bar{c}/\bar{s}$ for a pure I-131 source. This varies from 0.70 to 0.48 with foils from 2.0 to 3.5 mils thick
- r_s = $\bar{c}/\bar{s}$ for a pure S-35 source. This varies from 0.021 to 0.003 with foils from 2.0 to 3.5 mils thick
- r_d = $\bar{c}/\bar{s}$ for the decay standard planchette made as described in section 9
- r = $\bar{c}/\bar{s}$ for the unknown planchette
- x, x_d = counts of I-131 on the unknown and decay standard planchettes, respectively
- y, y_d = counts of S-35 on the unknown and decay standard planchettes, respectively
- T_s = total S-35 counts in the pertinent indicator added in step 2
- B = moles of the pertinent S-35 indicator added in step 2
- C_r = counts obtained without filter per mole of I-131 reagent used in step 1

On any planchette $\bar{s} = x + y$ and $\bar{c} = r_i x + r_s y$. Therefore $x/y = (r - r_s)/(r_i - r)$. For the decay standard $x_d/y_d = (r_d - r_s)/(r_i - r_d)$. Now $T_s = By_d/b$; $C_r = x_d/a$ and the amount of unknown, u, in moles $u = xT_s/yC_r = (x/y)(y_d/x_d)\cdot(a/b)\,B$ and in terms of the measured ratios this becomes $u = (r - r_s)(r_i - r_d)(a/b)B/(r_i - r)\cdot(r_d - r_s)$.

In preparing the decay standard planchette a/b should be chosen to bring r_d near the average of r_s and r_i. Since the counts due to I-131 disappear more rapidly than those due to S-35 it is useful to prepare standards with new ratios of a/b as the I-131 decays.

Alternative Standardizations.—Separate decay standard planchettes may be prepared from the I-131 pipsylalanine standardized on the Beckman and from each of the indicator S-35 solutions. If accurately known volumes are used in preparing these planchettes, the ratio of counts in one of these standards to the total S-35 counts added in the indicator is the ratio of the standard volume to the volume of the indicator solution used. This method makes a Beckman reading on the indicators unnecessary but requires that the standards reproduce the geometry of the unknowns. In using this method several standards are prepared from the same solution and compared to detect errors. If carefully handled, the standards are useful during the life of the S-35 preparations.

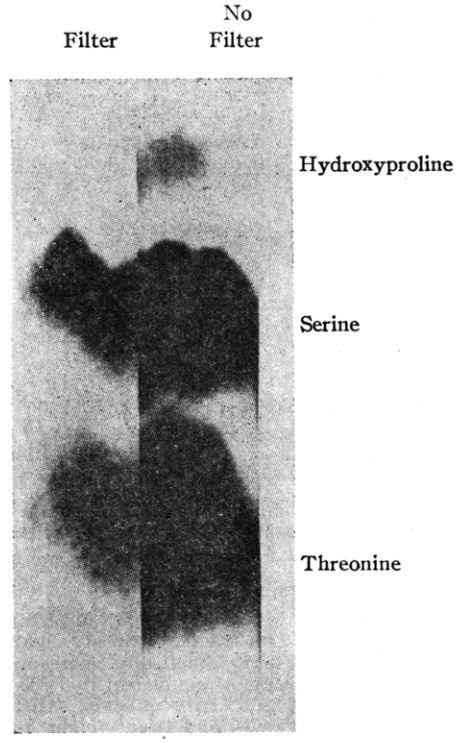

Fig. 1.—Radioautograms of a mixture containing no hydroxyproline in the unknown. The mark of hydroxyproline on the unfiltered side is produced by the S-35 indicator added. The marks of the serine and threonine bands demonstrate the presence of both I-131 and S-35.

Tests of the Method

A mixture containing the following amino acids was prepared. Glycine 50 mg., alanine 100 mg., valine 50 mg., leucine 50 mg., isoleucine 50 mg., proline 50 mg., phenylalanine 100 mg., tyrosine 50 mg., tryptophan 50 mg., methionine 50 mg., cystine 16 mg., arginine 50 mg., histidine 50 mg., lysine 50 mg. To this mixture the amino acids of groups I were added excepting one. An estimation of this one was then made on the mixture. These zero controls demonstrate the specificity of the method and the absence of the amino acids determined in the other constituents of the mixture. The amounts recorded in Table II are maximal. No attempt was made to obtain constant $\bar{c}/\bar{s}$ ratios since the results were, in all cases, well below the experimental error of the recovery experiments. In recovery experiments known amounts of the amino acids of interest were added and estimated. The amounts added and the average amounts recovered are shown in Table II. The indicated accuracy in duplicate experiments is 2%. This probably can be improved by longer counting periods. When the I-131 in adjacent bands occurs in

very disproportionate amounts it is sometimes advisable to elute each band from the chromatogram and to chromatograph each again. This procedure was used to purify the hydroxyproline bands in some of the protein hydrolysates.

TABLE II

RECOVERY OF GROUP I AMINO ACIDS FROM A MIXTURE (TOTAL 1000 MICROGRAMS)

Amino acid[a]	Added micrograms	Found micrograms
Glutamic acid	0	0.01
	99.3	98.0
Aspartic acid	0	0.07
	50.5	51.8
Hydroxyproline	0	0.00
	37.7	38.1
Serine	0	0.03
	49.6	48.3
Threonine	0	0.00
	33.6	33.8

[a] For other constituents of the mixture see text. In the zero added experiments each of the other listed amino acids was present.

Protein Analyses

The protein samples were dried at 105° and hydrolyzed in 6 *N* hydrochloric acid for twenty or twenty-eight hours. The acid hydrolysates were practically neutralized and made up to volume. Aliquots equivalent to 0.800 mg. of protein were analyzed as described above. There were no differences in the twenty and twenty-eight hour hydrolysates with respect to glutamic and aspartic acids. Serine and threonine were estimated only in the twenty hour hydrolysates and the results are not corrected for possible destruction during hydrolysis. The hydroxyproline values for the serum albumins were obtained after a second chromatographing of the original hydroxyproline bands. The β-lactoglobulin hydroxyproline band was not rechromatographed. The value 0.05% represents less than 0.1 residue per molecule and can be taken as an indication of the absence of this amino acid from the protein. The sample of β-lactoglobulin was prepared by Dr. R. K. Cannan. The human serum albumin (No. 42) and bovine serum albumin (L 18) were kindly supplied by Dr. Erwin Brand.

TABLE III

GROUP I AMINO ACIDS IN SEVERAL PROTEINS

Amino acid	Grams of amino acid per 100 grams of protein: β-Lactoglobulin[b]	Bovine serum albumin[b]	Human serum albumin[c]
Glutamic acid	18.2	16.3	17.0
Aspartic acid	10.5	9.6	8.95
Hydroxyproline	<0.05	<0.0006	<0.0007
Serine[a]	3.85	4.03	2.97
Threonine[a]	4.95	5.49	4.35

[a] These values are not corrected for loss in hydrolysis. [b] For literature values *cf.* W. H. Stein and S. Moore, *J. Biol. Chem.*, **178**, 79 (1949). [c] For literature values *cf.* E. Brand, *Ann. N. Y. Acad. Sci.*, **47**, 187 (1946).

Preparations

S-35 Pipsyl Chloride.—A modification of the procedure of Stoll and Stutzer[10] is used to prepare acetylsulfanilic acid from acetanilide. It was noted[11] that practically quantitative recovery of sulfuric acid was obtained by using a mixture of acetic anhydride and acetic acid as the solvent whereas each alone gave very low yields. Pure acetic anhydride gave a discolored product in low yield.

The S-35 is obtained as a dilute solution of sulfuric acid[12] which is evaporated to a volume of 0.5 ml., transferred to a small glass stoppered tube, mixed with 40 mg. of non-isotopic sulfuric acid and evaporated until the first fumes appear. After cooling, 0.7 ml. of glacial acetic acid, 0.3 ml. of fresh acetic anhydride and 80 mg. of acetanilide are added. The stoppered tube is warmed to 60° for two hours and then kept at room temperature overnight. The mixture is diluted with 10 ml. of water and 2 ml. of concentrated hydrochloric acid. By heating for two hours on the water-bath the acetylsulfanilic acid is hydrolyzed. After cooling the solution on ice, a several-fold excess of sodium nitrite is added to form diazobenzenesulfonic acid. After ten minutes, excess nitrous acid is removed by adding ammonium sulfamate. An excess of potassium iodide is then added and the mixture is cooled and 100 mg. of non-isotopic *p*-iodobenzenesulfonic acid added along with enough sodium chloride to saturate the solution. The sodium salt of *p*-iodobenzenesulfonic acid precipitates and is removed by centrifugation. The mother liquors are cleared of S-35 derivative by adding a second portion of 100 mg. of non-isotopic *p*-iodobenzenesulfonic acid, dissolving by warming and allowing to cool and precipitate. Both batches of precipitate are combined and converted to chloride as described for the I-131 labeled derivative.[4b]

S-35 Pipsylamino Acids.—Small amounts of the required indicator amino acids are conveniently made by a single step of the process described for quantitative pipsylation of unknowns,[4b] except that a considerable excess of amino acid (30 mg.) over S-35 pipsyl chloride (20 mg.) is used. Combined ether extracts of the reaction mixture are treated with ammoniacal ether whereupon the ammonium salts of the pipsylamino acids precipitate. The salts, ammonium pipsylaspartic acid and ammonium pipsylglutamic acid, precipitate as oils. The precipitates are dissolved in small volumes of water and precipitated as crystalline acids by addition of hydrochloric acid. The precipitates are finally dissolved in dilute ammonia and diluted to a concentration of 0.002–0.004 *M*. The actual concentration is determined by the absorption at 250 mμ. as measured in acid solution in a Beckman D. U. spectrophotometer after suitable dilution. The solutions of indicators have kept for several months in the refrigerator. Their purity is checked by chromatographing and demonstrating one band only with a suitable developer.

Discussion

It is evident that the indicator principle is general and that the principles of analysis implicit above are applicable to the estimation of any compound from which a suitable isotopic derivative may be formed. It may be pointed out at this time that if a known amount of a labeled compound of interest is added to an unknown mixture the isotopic derivative method becomes independent of quantitative yield in the derivative formation. Thus if glycine containing C-14 were added to a protein hydrolysate and the mixture pipsylated with I-131 reagent, the fractional recovery of C-14 in the purified pipsylglycine would demonstrate the fractional recovery of the non-isotopic glycine as well. Development of this principle in in progress.

The grouping procedure considerably simplifies subsequent chromatography. For instance pipsylalanine, pipsylthreonine and pipsylphenylalanine have nearly the same retardation factors as ammonium salts with pentanol or butanol as the developer. They fall, however, into different groups in Table I with threonine in group

(10) Stoll and Stutzer, *Ber.*, **42**, 4539 (1909).

(11) R. A. Lichtin, Master's Thesis, New York University, N. Y., 1945.

(12) A. E. C. catalog item S44.

I, alanine in group II and phenylalanine (which goes into CCl_4 from acid) in group III.

Interesting qualitative demonstrations can be prepared by radio autographing the chromatograms in a sandwich made of two sensitive films, a suitable filter and the chromatogram. The filter is chosen to eliminate S-35 radiation but permit most I-131 radiation to pass through. Any band registered only on the unfiltered side demonstrates the absence of I-131 in it and therefore the absence of the amino acid in question from the sample. A pair of radioautographs showing the absence of hydroxyproline is shown in Fig. 1. The same pair shows the presence of serine and threonine.

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Summary

A method is described for the estimation of glutamic acid, aspartic acid, hydroxyproline, serine and threonine in 0.2–1.0 mg. of hydrolyzed protein. Paper chromatography is applied to the separation of the *p*-iodobenzenesulfonyl derivatives formed from the protein hydrolysate products with a reagent containing I-131. The recovery is measured by the use of indicators consisting of known *p*-iodobenzenesulfonyl derivatives containing S-35 which are added in known amounts immediately after forming the I-131 derivatives of the amino acids. Radioautograms are used to locate the bands and constancy of I-131 to S-35 ratios in successive portions of the bands provides a test of the validity of the analysis.

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[CONTRIBUTION FROM THE RESEARCH DEPARTMENT OF EATON LABORATORIES, INC.]

Furans. II. Ultraviolet Absorption and Molecular Structure

BY ROBERT F. RAFFAUF

In recent years absorption spectra have assumed an increasing importance as an approach to the problems of structural organic chemistry. Although a number of observations on the spectrochemistry of furans have appeared in the literature, much remains to be learned concerning the relationship between the absorption characteristics and the molecular structure of these compounds. The present work was undertaken in order to discover any such general relationships in a series of compounds useful to a more comprehensive study of the chemical and physiological properties of the nitrofurans which has been in progress in this Laboratory. The results are summarized in Tables I and II.

TABLE I

R = 2-furyl

Compound	M. p., °C.	Max. mμ	ϵ[a]
R—H	...	<220	...
R—CH_2OH	...	<220	...
R—CHO	B. p. 160	230	3500
		275	13750
R—$COCH_3$	30–31	225	3000
		275	13900
R—$COOC_2H_5$	34–35	255	13250
R—CH=NNHCONH$_2$	197–198	290	25000
Furan 2,5-dialdehyde	109–110	290	16900
5-Bromofurfuralsemicarbazone[b]	185 d.	300	27500
5-Methylfurfuralsemicarbazone	195–197	300	24850

[a] In distilled water. [b] Calcd. for $C_6H_6BrN_3O_2$: C, 31.0; H, 2.58. Found: C, 31.3; H, 2.72. *Cf.* Scheibler, *J. prakt. Chem.*, **136**, 232 (1933).

TABLE II

R = 5-NITRO-2-FURYL

Compound	M. p., °C.	Max. mμ	ϵ[a]
R—H[f]	26–28	225	3400
		315	8100
R—CH_2OH[g]	B. p. 145 (5 mm.)	230	3750
		320	10350
R—CHO[h]	34–37	225	8250
		310	11600
R—$COCH_3$[i]	76–77	225	10200
		310	11500
R—$COOC_2H_5$[j]	102–104	<200	...
		305	10900
R—NO_2[k]	101.5–102	230	7500
		310	11500
R—CH=NNHCONH$_2$[l]	238 dec.	269	13200
		375	15800
R—C(CH_3)=NNHCONH$_2$[i]	248–250 dec.	260	13250
		375	14000
R—CH=NN(CH_3)COHN$_2$[b,m]	213–214	265	13600
		385	16100
R—CH=NNHCONH—CH_3[c,m]	201–202 dec.	265	12600
		380	15700
R—C(CH_3)=NN(CH_3)CONH$_2$[e,m]	187–190 dec.	225	12100
		320	9250
		360	8100

[a] In distilled water. [b] Calcd. for $C_7H_8N_4O_4$: C, 39.6; H, 3.79. Found: C, 39.8; H, 3.63. [c] Calcd. for $C_7H_8N_4O_4$: C, 39.6; H, 3.79. Found: C, 39.9; H, 3.51. [e] Calcd. for $C_8H_{10}N_4O_4$: C, 42.5; H, 4.68. Found: C, 42.9; H, 4.43. [f] Marquis, *Comp. rend.*, **132**, 141 (1901); Marquis, *Ann. chim. phys.*, [8] **4**, 216 (1905). [g] Gilman and Wright, THIS JOURNAL, **53**, 1924 (1931). [h] Gilman and Wright, *ibid*, **52**, 2550, 2552, 4165 (1930); Gilman and Wright, *Rec. trav. chim.*, **50**, 834 (1931). [i] Rinkes, *Rec. trav. chim.*, **51**, 349 (1932). [j] Marquis, *Compt. rend.*, **135**, 506 (1902). Freure and Johnson, THIS JOURNAL, **53**, 1142 (1931). [k] Hill and White, *Am. Chem. J.*, **27**, 193 (1902). [l] U. S. Patent 2,416,234. [m] These laboratories.