uct was extracted with ether. The ether solution was washed with water till neutral and dried over sodium sulfate. Upon removing the ether 192 mg. of nicely crystalline product was recovered, m. p. 118–121°. When recrystallized from a small volume of dilute acetone it melted at 124–125° and did not depress the m. p. of an authentic sample of V.

Anal. Calcd. for $C_{22}H_{34}O_2$: C, 79.94; H, 10.37. Found: C, 79.68; H, 10.45.

Pregnene-3β-ol-20-one Acetate (VI).—80 mg. of pregnene-3β-ol-20-one methyl ether and 40 mg. of *p*-toluene sulfonic acid monohydrate was covered with 4 ml. of redistilled acetic anhydride and heated on a steam cone with stirring for one-half hour. The mixture was cooled and poured into ice-water with stirring, and allowed to stand two hours. The product was extracted with ether, washed with sodium bicarbonate solution and water and dried over sodium sulfate. Upon removing the solvent and recrystallizing the residue three times from dilute methanol the product melted at 143–144° and did not depress the melting point of an authentic sample of VI.

Summary

1. The secondary carbinol, 22-phenyl-3-methoxy-22-hydroxy-bisnor-5-cholene (III), has been dehydrated in yields of 60–75% and the resulting diene (IV) has been degraded to give pregnene-3β-ol-20-one methyl ether (V).

2. The mono tosylate mentioned previously[1] has been shown to be 22-phenyl-3,22-dihydroxy-bisnor-5-cholene-3-tosylate (II).

KALAMAZOO, MICHIGAN RECEIVED AUGUST 19, 1948

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

A Method for the Determination of Organic Compounds in the Form of Isotopic Derivatives.[1,2] I. Estimation of Amino Acids by the Carrier Technique

BY ALBERT S. KESTON, SIDNEY UDENFRIEND[3] AND R. KEITH CANNAN

In a preliminary communication[4] we have outlined a general method for the estimation of organic compounds in the form of isotopically labelled derivatives. The method is of particular value for the estimation of the components of mixtures of related compounds for which individual chemical methods of analysis are not available. We have applied it to the estimation of certain of the amino acids which are present in hydrolysates of proteins.

The mixture is treated with a reagent containing a stable or a radioactive isotope under such conditions that the components which are to be estimated are quantitatively converted into derivatives of the reagent. If any one of these derivatives can be separated quantitatively from the others, it can then be directly estimated[5] with the precision and sensitivity characteristic of isotopic measurements. In the analysis of mixtures of amino acids, however—and, indeed, of most biological materials—the quantitative separation of a single component is not always attainable. In this situation, the isotopic method may be applied with the aid of carrier or indicator techniques.[4,5]

In the carrier method, an overwhelming excess, W moles, of the unlabelled derivative of the desired constituent is added to the mixture and is then separated and purified to constant molal isotope concentration, Cc. If Cr be the molal isotope concentration of a pure isotopic derivative which has been prepared with the same sample of the reagent, then the amount of the isotopic derivative (w) which was present in the mixture is

$$w = Cc(w + W)/Cr$$

When relatively large amounts of the carrier are added, this equation reduces to

$$w = WCc/Cr$$

The carrier principle gives the method wide scope and flexibility. It makes it possible to carry out estimations of very minute amounts of the compound of interest and yet to operate with milligram quantities of material. It is, moreover unnecessary to seek a quantitative recovery of the compound in pure form. What is essential is the rigorous purification of the carrier from significant amounts of isotopic impurities. To accomplish this large losses of the product may be accepted. Finally, in the analysis of optically active compounds, the use of a large excess of a racemic carrier ensures the estimation of the total of both of the active forms. This is a significant advantage in the analysis of proteins in which indeterminate degrees of racemization may have occurred during hydrolysis. If, on the other hand, one wishes to estimate only one of the isomers the corresponding carrier may be used.

The validity and precision of the method which has been outlined depend primarily on, (a) the completeness of the reaction between the isotopic reagent and the compound which is to be estimated, (b) the rigorous purification from radioactive contaminants of the carrier after addition to and isolation from the reaction mixture and (c) the precision of the measurements of Cc and Cr.

(1) Taken from a thesis submitted in December, 1947, by Sidney Udenfriend to the Faculty of the Graduate School in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Chemistry.

(2) This work was done under a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

(3) Present address: Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Mo.

(4) A. S. Keston, S. Udenfriend and R. K. Cannan, THIS JOURNAL, **68**, 1390 (1946).

(5) A. S. Keston, S. Udenfriend and M. Levy, *ibid.*, **69**, 3151 (1947).

In this communication, we describe in detail the application of the carrier technique to the estimation of glycine, alanine and proline in hydrolysates of proteins. The reagent which we have used is *p*-iodophenylsulfonyl chloride (pipsyl chloride) containing I^{131}. The evidence that the above analytical criteria are met in the estimation of these amino acids in mixtures will first be reviewed.

The Reaction of Pipsyl Chloride with Amino Acids.—In preliminary investigations[4] the amino acid was dissolved in a solution of carbonate and treated with a substantial excess of pipsyl chloride. The progress of the reaction was followed by the rate of disappearance of amino nitrogen as indicated by Folin's naphthoquinone reaction.[6] When all of the amino nitrogen had disappeared it was assumed that the sole product was the monosubstituted sulfonamide. In some later experiments, however, the presence of a small amount of a second radioactive product was observed. These products, which were obtained from alanine and isoleucine but not from glycine or proline, were well-defined crystalline substances, highly insoluble in water both as free acids and as potassium salts. They have not been rigorously identified but, since they have been found to contain two atoms of labelled iodine per atom of nitrogen, we may assume that they are the disubstituted sulfonamides (dipipsyl derivatives).

The attempt was made to drive the secondary reaction to completion with the object of using the dipipsyl derivatives as carriers. Good yields were obtained when a solution of aniline or of isoleucine in saturated potassium carbonate was shaken with a large excess of pipsyl chloride dissolved in acetone.[7] Under the best conditions, however, the secondary reaction attained only about 90% completion. The attempt to use the dipipsyl derivatives as carriers was, therefore, abandoned.

Re-investigation of the over-all reaction indicated that the second stage occurs to a significant extent only after substantial completion of the first. When a series of successive partial reactions is carried out with intermittent removal of the monopipsyl compound formed it has been possible, consistently, to recover 98–100% of the amino acid as the monopipsyl derivative. This manner of carrying out the reaction has been adopted as the standard analytical procedure. Mono- and dipipsyl carriers have been used to estimate the amounts of these two types of derivative which are formed under the standard conditions (Table I). In our experience, so little dipipsyl material is formed in a protein hydrolysate that the use only of a monopipsyl carrier gives results of sufficient accuracy. If desired, of course, a second estimation may be made using the dipipsyl carrier and correcting the monopipsyl estimation for any dipipsyl compound which may be found. Since proline and hydroxyproline are secondary amines, they cannot form dipipsyl compounds. Apparently glycine does not readily do so.

(6) O. Folin, *J. Biol. Chem.*, **51**, 377 (1922).

(7) The aqueous and acetone phases were immiscible in the presence of the high concentration of carbonate.

The Purification of the Carriers.—The radioactive impurities which may be expected to be present in a protein hydrolysate which has been treated with pipsyl chloride are the monopipsyl derivatives of the amino acids together with traces of the dipipsyl compounds of some of them, pipsyl amide arising from reaction with ammonia and *p*-iodophenyl sulfonic acid which is formed by the hydrolysis of the excess of pipsyl chloride. The pipsyl amino acids are readily extracted by ether from acid solutions and return to the aqueous phase when shaken with dilute ammonia. The iodophenyl sulfonic acid is not readily extracted by ether from dilute acid, whereas the pipsyl amide remains in the ether when shaken with ammonia. Thus the removal of the major radioactive contaminants from the pipsyl amino acids presents no great difficulty.

TABLE I

RECOVERY OF AMINO ACIDS AS THE MONOPIPSYL AND DIPIPSYL DERIVATIVES[a]

Amino acid	Amount added, mg.	% recovered as monopipsyl	% recovered as dipipsyl	Total, %
Glycine	1.000	100.2	...[b]	100.2
	1.000	99.8	...[b]	99.8
D,L-Alanine	1.000	98.7	1.3	100.0
	1.000	99.1	1.2	100.3
D,L-Isoleucine	2.000	98.0	1.5	99.5
L-Proline	3.232	98.6	...[c]	98.6

[a] These recoveries are from analyses of the individual pure amino acids, using the standard procedure. [b] No dipipsyl derivative of glycine could be isolated for use as carrier. [c] Proline is a secondary amine and can form only the monosubstituted derivative.

A more difficult problem has been the separation of the carrier from traces of other pipsyl amino acids. We have found alternate repeated crystallization and treatment with charcoal to be effective though sometimes tedious. The free pipsyl amino acids are poorly soluble. Consequently, crystallization is readily carried out by acidification of an ammoniacal solution. The crystals may then be dissolved again in ammonia, treated with charcoal and recrystallized. This cycle must be repeated very many times before constant isotopic concentration is attained. The pipsyl derivatives of leucine and isoleucine, for example, are very difficult to separate from one another by crystallization. The purification of pipsylalanine in the presence of pipsylglycine or pipsylproline has also proved to be very tedious if crystallization alone is relied on.

The pipsyl amino acids, like the amino acids themselves (unpublished data), evidently have a strong tendency to co-precipitate. We have found that the phenomenon results in a distribution between the solid and the dissolved carrier as though the impurities were distributing between

two immiscible solvents. The distribution co-precipitation constant k equals

$$\frac{\left(\frac{\text{Amount of impurity}}{\text{Amount of carrier}}\right)_{\text{Supernatant}}}{\left(\frac{\text{Amount of impurity}}{\text{Amount of carrier}}\right)_{\text{Crystals}}} = k$$

The absolute amount of the impurity has little effect on the magnitude of the distribution constant. Dilution of the isotopic impurity with its nonisotopic analog is thus of little value. The value of k and the amount sacrificed determine the degree of purification in a crystallization. The various pipsyl amino acids have individual characteristic distribution coefficients in the presence of a particular carrier. The coefficients can be determined in simple experiments and are helpful in determining which particular pipsyl amino acid impurities will be difficult to remove from a given carrier.

When this type of distribution obtains, the efficiency of purification in relation to the amount of material lost increases as the number of crystallizations over which the loss is spread is increased. If the distribution coefficient is unfavorable, the asymptotic approach to isotopic purity is very slow. We accept a carrier as pure only when its isotope concentration has remained constant, within the error of measurement, through several recrystallizations and treatments with charcoal so conducted that there have been large losses of carrier.

The tedium of crystallization in some cases made it desirable to find other methods of purification. The work of Martin and Synge[8,9,10] on the distribution of the N-acetyl amino acids between solvent pairs was suggestive. Accordingly, we determined approximate distribution coefficients of the pipsyl amino acids between selected pairs of solvents. Representative results are given in Table II where the coefficient is expressed as the concentration in the organic phase divided by that in the aqueous phase.

The coefficients in Table II show wide characteristic differences. It is interesting to note from columns 4 and 5 that the relative coefficients of the pipsyl amino acids in a chloroform–water system closely parallel those of the acetyl amino acids. The range of distribution coefficients is so wide that some groups of pipsyl amino acids can be effectively separated from others by simple extraction. The leucine derivatives, for example, can be separated from those of the dicarboxylic acids by extracting an acid solution of the mixture with carbon tetrachloride.

Advantage may be taken of smaller differences in distribution by the use of an appropriate form of counter-current separation. For the separations with which this paper is concerned, we have found that a ten-plate discontinuous counter-current process of the type exploited by Craig[11] is adequate. The conduct of such a separation is described in a later section. From the crude fractions which are obtained, pure samples of the pipsyl derivatives of glycine, alanine and proline are readily obtained by crystallization. The adoption of this process of preliminary purification has made it possible to carry out several estimations on a single aliquot of a protein hydrolysate by adding several carriers to it prior to counter-current separation. Glycine, alanine and proline can be conveniently estimated in this way. We have also used the counter-current technique to ascertain the isotopic homogeneity of supposedly purified carriers.

The traces of dipipsyl derivatives which are formed in the reaction with pipsyl chloride tend to accumulate in the proline fraction of the counter-current process. They are difficult to remove by crystallization. The dipipsyl derivatives show a notable tendency to co-precipitate. We have been able to expedite the purification of pipsylproline by taking advantage of this characteristic. A small amount of sodium dipipsylalanine is added to an ammoniacal solution of the pipsylproline fraction and is then precipitated by addition of potassium chloride. This removes much of the radioactivity impurity in the fraction and facilitates final purification of the pipsylproline by crystallization.

Measurements.—Two measurements are made on an aliquot of the carrier at each stage of purification until constant specific activity is

TABLE II

DISTRIBUTION COEFFICIENTS OF SEVERAL PIPSYL AMINO ACIDS

Column I. Distribution coefficients of pipsyl derivatives between normal butanol and 0.2 *M* sodium hydroxide. Column II. Distribution coefficients of pipsyl derivatives between chloroform and 0.2 *M* hydrochloric acid. Column III. Distribution coefficients of pipsyl derivatives between carbon tetrachloride and 0.2 *M* HCl. Column IV. Relative partition coefficients of pipsyl derivatives between chloroform and 0.2 *M* HCl; pipsylglycine as unity. Column V. Relative partition coefficients of the N-acetyl derivatives between chloroform and water; acetylglycine as unity.

Amino acid	I	II	III	IV	V[a]
Glycine	0.14	0.67	0.006	1	1
Alanine	0.13	3.25	0.04	4.8	3.5
α-Aminoisobutyric acid	0.59	7.0	0.11	10.5	...
Valine	0.60	20.0	0.40	30	20
Leucine	1.5	43	1.2	64	85
Isoleucine	1.7	50	1.4	75	100
Serine	0.06	0.04	0.00	0.06	0.6
Threonine	..	...	0.00	...	...
Methionine	0.37	...	0.26	...	27
Proline	..	28	...	40	40
Aspartic acid	0.01	0.04	0.00	0.06	...
Glutamic acid	0.02	0.04	0.00	0.06	0.6

[a] R. L. M. Synge, *Biochem. J.*, **33**, 1913 (1–4) (1939).

(8) A. J. P. Martin and R. L. M. Synge, *Biochem. J.*, **35**, 91 (1941).
(9) A. J. P. Martin and R. L. M. Synge, *ibid.*, **35**, 1358 (1941).
(10) R. L. M. Synge, *ibid.*, **33**, 1913 (1–4) (1939).
(11) L. C. Craig, *J. Biol. Chem.*, **155**, 519 (1944).

TABLE III

PROPERTIES OF PIPSYL DERIVATIVES OF SEVERAL AMINO ACIDS

Pipsyl amino acid	Melting point (cor., °C.)	Mol. extinct. coeff. at 250 mμ × 10⁻³	Neutralization equivalent Found	Neutralization equivalent Theoretical
Glycine	205–205.5	15.9	339.6	341.0
DL-Alanine	194.5	15.8	355.3	355.0
D-Alanine	...	15.8	356.2	355.0
Aminoisobutyric acid	183.0	..	372.0	369.1
DL-Valine	181.5	..	...	...
DL-Leucine	125.5–126.5	..	...	...
DL-Isoleucine	149.0	15.8	397.6	397.0
DL-Serine	209.5 (dec.)	15.7	...	...
DL-Methionine	127.0	..	416.7	416.0
DL-Proline	...	16.5	...	...
L-Proline	126.0	16.5	384.0	382.0
DL-Aspartic acid	182.0–184.0	..	..	...

attained. The first is a measurement of the amount of carrier which remains. This may be done conveniently on a very small sample by measuring the absorption at 250 mμ (Table III). The second is a measurement of radioactivity. We use a double-walled thin glass cylinder[12] which is slipped over a Geiger–Müller counter filled with tetramethyllead vapor[13] together with a Technical Associates model GS-4 scaling unit.

sis. This corrects for radioactive decay. The standard is purified by extraction into ether and re-extraction into dilute alkali followed by recrystallization. A solution of the purified material is prepared, standardized photometrically and used in all assays.

The spectrophotometric measurements may be made with an accuracy better than 0.5%. The measurement of radioactivity, when carried out as described, is capable of a high degree of accuracy. The probable error due to randomness of counting is equal to $1.67\sqrt{A + 2B}$, where A is the number of counts of the sample and B is the background count. We have routinely relied on observations involving 5,000–10,000 counts in a two-minute period. With a background count of 20 the error due to randomness is about 1%. Several two-minute counts have been averaged to reduce this error.

The procedure which is described in the section headed "Analytical Procedure" has been applied to the estimation of glycine, alanine and proline in a mixture of pure synthetic amino acids (Table IV) and to the acid hydrolysates of four crystalline proteins for which some comparative analytical data are available (Tables V–VIII). Control estimations have also been carried out on amino acid mixtures from which the particular amino acid to be estimated was omitted.

In the absence of the amino acid, it has in all

TABLE IV

RECOVERY OF ADDED AMOUNTS OF GLYCINE, ALANINE AND PROLINE FROM A MIXTURE[a] OF AMINO ACIDS

Added γ	Glycine carrier remaining at various stages of purification %	Amino acid found at corresponding stages γ	Added γ	Alanine[b] carrier remaining at various stages of purification %	Amino acid found at corresponding stages γ	Added γ	Proline carrier remaining at various stages of purification %	Amino acid found at corresponding stages γ
0.00	40.5	24.5	0.00	15.6	18.5	0.00	27.5	4.60
	30.0	12.0		12.2	5.0		20.6	0.48
	17.6	4.2		10.0	3.0		16.5	0.00
	11.1	1.2		8.4	1.55			
	9.1	0.11		7.5	0.67			
				6.2	0.08			
50.0	17.9	50.5	99.5	24.1	97.5	80.8	42.0	81.1
	14.7	50.1		18.4	96.4		30.5	82.9
	11.3	50.6		14.7	99.3		19.5	80.6
	Average	50.4		Average	97.7		Average	81.5
50.0	18.5	51.0	99.5	23.9	102.1	80.8	42.0	81.1
	13.8	50.7		19.8	102.1		33.1	81.7
	9.9	50.4		16.3	99.6		23.1	80.9
	Average	50.7		Average	101.3		Average	81.2

[a] The amino acid mixture contained 150 μg of leucine and of lysine, 110 μg of glutamic acid, 100 μg of isoleucine and of phenylalanine, 99.5 μg of alanine, 80.8 μg of proline, 50 μg of threonine, of glycine and of valine and 25 μg each of methionine and aspartic acid. In all cases (except glycine) the synthetic racemic forms were used. In the control analyses the glycine, alanine and proline were omitted from the mixture. [b] The value for alanine is corrected for 1.3% dipipsylalanine.

With each set of determinations of the unknown counts are also made of a standard solution of an isotopic monopipsyl derivative prepared from the same radioactive reagent as is used in the analy-

cases been possible to purify the carrier to an isotopic concentration corresponding to a negligible content of the amino acid in the mixture analyzed. In the presence of the amino acid, very satisfactory recoveries have been recorded on mixtures of known composition.

(12) E. Chargaff, *J. Biol. Chem.*, **128**, 379 (1939).

(13) A. S. Keston, *Rev. Sci. Inst.*, **14**, 295 (1943).

TABLE V

ANALYSIS OF β-LACTOGLOBULIN, 15.6% NITROGEN[a]

Amino acid	Amount of protein employed, mg.	Amount of carrier added, mg.	Carrier remaining, %	Grams of amino acid per 100 g. of protein	Values in the literature
Glycine	1.319	200.0	11.5	1.62	
			8.2	1.56	
			5.6	1.57	
			Average	1.58	
	1.319	200.0	14.7	1.49	1.5[d]
			11.7	1.57	1.4[e]
			8.6	1.56	
			Average	1.54	
Alanine	1.319	300.0	13.5	7.14	
			7.9	7.09	
			6.5	7.11	6.64[e]
			Average	7.11	
	2.638	300.0	22.0	6.87	6.2[a]
			18.4	6.96	
			14.3	7.09	6.2[f]
			9.9	7.08	
			Average	7.00	
D-Alanine[b]	2.000	200.0	7.4	0.1	
Proline (total)	2.638	200.0	49.4	4.74	
			41.8	4.80	
			31.8	4.82	
			Average	4.79	
	2.638	200.0	48.8	4.96	4.1[a]
			41.1	4.80	
			35.6	4.90	5.4[f]
			Average	4.89	
L-Proline[c]	2.638	200.0	16.1	4.86	
			10.4	4.94	
			6.7	4.83	
			Average	4.88	

[a] E. Brand, L. J. Saidel, W. H. Goldwater, B. Kassel and F. J. Ryan, THIS JOURNAL, 67, 1524 (1945). [b] The pipsyl derivative of D-alanine was used as carrier in this determination. [c] The derivative of the natural form of proline was used as carrier in this case. [d] G. L. Foster, *J. Biol. Chem.*, 159, 431 (1945). [e] A. C. Chibnall, *J. Intern. Soc. Leather Trades' Chemists*, 30, 1 (1946). [f] G. R. Tristram, *Biochem. J.*, 40, 721 (1946).

The four proteins were hydrolyzed in 6 *M* hydrochloric acid for about twenty hours in sealed tubes at 110–120°. The amount of protein was based on the weight of an air-dried sample and corrected for the moisture which was determined by drying to constant weight at 110°. The hydrolysates were partially neutralized and diluted to contain about 5–10 mg. protein per ml. For analysis, an aliquot was measured with a constriction micro pipet,[14] transferred to a Folin sugar tube, neutralized and analyzed by the procedure described below.

The alanine and proline carriers were prepared from the DL amino acids except in two cases noted

(14) M. Levy, *Compt. rend. Lab. Carlsberg*, **21**, No. 6, 101 (1936).

TABLE VI

ANALYSIS OF HUMAN HEMOGLOBIN, 16.9% NITROGEN[a]

Amino acid	Amount of protein employed, mg.	Amount of carrier added, mg.	Carrier remaining, %	Grams of amino acid per 100 g. of protein	Values in the literature
Glycine	2.056	160.0	7.6	4.52	
			5.7	4.44	
			4.0	4.42	
			Average	4.46	
	2.056	160.0	21.6	4.48	
			16.9	4.54	
			13.2	4.44	
			Average	4.49	
Alanine (total)	2.056	300.0	23.3	10.20	
			17.3	9.75	
			13.2	10.30	
			Average	10.08	
	2.056	300.0	25.2	9.85	9.9[b]
			19.1	9.70	
			15.9	9.90	
			Average	9.82	
Proline (total)	2.056	160.0	57.8	5.06	
			49.1	4.88	
			37.1	4.93	
			Average	4.96	
	2.056	160.0	42.3	4.90	
			30.7	5.02	
			25.1	4.85	
			Average	4.92	
Isoleucine	2.000	200.0	..	<0.2	0.7[c]
					0.30[d]
	2.000	200.0	..	<0.5	0[e]

[a] H. B. Vickery, *J. Biol. Chem.*, **144**, 719 (1942). [b] J. Roche and M. Mourgue, *Trav. membres soc. chim. biol.*, **23**, 1329 (1941). [c] A. A. Albanese and T. M. Barnes, *J. Biol. Chem.*, **157**, 613 (1945). [d] H. B. Devline and C. A. Zittle, *J. Biol. Chem.*, **156**, 393 (1944). [e] E. Brand and J. Grantham, THIS JOURNAL, 68, 724 1946).

in the tables. The isoleucine carrier used in the hemoglobin analysis was prepared from a sample of the synthetic product which was stated to be free from leucine (The Winthrop Chemical Co.). The analyses recorded in the tables for alanine include a correction of 1.3% for the amount of dipipsylalanine presumed to have been formed (Table I). No corrections have been applied to the glycine and proline results.

The samples of β-lactoglobulin[15] and of crystalline human hemoglobin[16] were prepared in this Laboratory. The crystalline preparations of aldolase and phosphoglyceric aldehyde dehydrogenase of rabbit skeletal muscle were supplied by Dr. S. F. Velick of Washington University, St. Louis.

In the analysis of β-lactoglobulin, the estima-

(15) A. H. Palmer, *J. Biol. Chem.*, **104**, 359 (1934).

(16) R. K. Cannan and J. Redish, "Blood Substitutes and Blood Transfusions," Charles C. Thomas, Publisher, 1942, p. 147.

TABLE VII

ANALYSIS OF ALDOLASE (RABBIT SKELETAL MUSCLE), 16.8% NITROGEN[a]

Amino acid	Amount of protein employed, mg.	Amount of carrier added, mg.	Carrier remaining, %	Grams of amino acid per 100 g. of protein	Values in the literature
Glycine	1.426	200.0	21.3	5.62	
			16.8	5.70	
			11.0	5.65	
			Average	5.66	6.12[b]
	1.426	300.0	30.0	5.55	
			24.0	5.55	
			19.0	5.56	
			Average	5.55	
Alanine	1.426	301.2	16.8	8.79	
			10.9	8.68	
			6.7	8.70	
			Average	8.72	7.87[c]
	1.426	301.2	26.2	8.43	
			20.7	8.43	
			14.5	8.48	
			Average	8.45	
Proline	1.426	160.0	20.5	5.90	
			13.3	5.85	
			9.6	5.92	
			Average	5.89	
	1.426	160.0	45.6	5.80	
			36.4	5.61	
			29.3	5.68	
			Average	5.69	

[a] S. F. Velick and F. Ronzoni, *J. Biol. Chem.*, 173, 627 (1948).

TABLE VIII

ANALYSIS OF PHOSPHOGLYCERALDEHYDE DEHYDROGENASE (RABBIT SKELETAL MUSCLE), 16.4% NITROGEN[a]

Amino acid	Amount of protein employed, mg.	Amount of carrier added, mg.	Carrier remaining, %	Grams of amino acid per 100 g. of protein	Values in the literature
Glycine	1.925	200.0	22.3	5.90	
			18.0	5.94	
			13.2	6.08	6.15[b]
			Average	5.97	
	1.925	200.0	19.7	6.13	
			16.0	6.10	
			9.0	6.10	
			Average	6.11	
Alanine	1.925	301.2	16.1	6.80	
			10.9	6.66	
			6.9	6.66	
			Average	6.71	5.92[c]
	1.925	301.2	16.6	6.64	
			11.1	6.81	
			7.7	6.82	
			Average	6.76	
Proline	1.925	160.0	29.0	3.65	
			20.8	3.58	
			16.1	3.57	
			Average	3.60	
	1.925	160.0	31.6	3.78	
			24.8	3.82	
			20.8	3.81	
			Average	3.80	

[a] S. F. Velick and F. Ronzoni, *J. Biol. Chem.*, 173, 627 (1948). Attention is drawn to an error in the text of this paper (p. 637). The glycine content of the dehydrogenase given as 6.88 per cent. by the isotopic derivative method should be corrected to 6.04 and the value for microbiological assay given as 6.98 per cent. should be corrected to 6.15. The correct values are those in the tables in the same article.

tion of total proline and of L-proline are in excellent agreement. The D-alanine estimation is a maximum one because constant isotope concentration was not attained. It is evident, however, that no significant amount was present in the hydrolysate. The same comment applies to the isoleucine estimation of human hemoglobin. This protein contains a large amount of leucine which, at the present stage of development of the method, is not easily separated from isoleucine.

Analytical Procedure

The procedure described below for the determination of glycine, alanine and proline in a single sample is given in detail to illustrate the use of more than one carrier.

To a Folin sugar tube, containing an amount of hydrolysate corresponding to 0.3–2 mg. protein, are added 15 mg. of sodium bicarbonate, 9 mg. of radioactive pipsyl chloride and water to a volume of 0.6 ml. The tube is placed in a boiling water-bath. When the pipsyl chloride has melted, the tube is agitated with an efficient vibrator for a few minutes. We have used an electrical massaging unit so attached to the ring stand holding the reaction tube as to produce high frequency-short amplitude vibrations sufficient to emulsify the pipsyl chloride and water.

After ten minutes the tube is cooled and acidified with 0.2 ml. of *M* hydrochloric acid. A glass bead and 2 ml. of ether are added and the pipsyl derivatives are extracted into the organic solvent. The ether is transferred by a rubber-bulb pipet to a vessel containing measured amounts (about 200 mg. each) of the appropriate carriers in ammoniacal solution. Three more extractions with ether are made and the extracts are transferred without loss to the carrier mixture. The contents of the sugar tube are reduced to dryness by heat and suction, 9 mg. of pipsyl chloride, 15 mg. of sodium bicarbonate and 0.6 ml. of water are added and the reaction is repeated as before. The mixture is acidified and extracted with ether and then is submitted to a third reaction with 12 mg. of pipsyl chloride.

The vessel containing the carriers and the combined ether extracts is warmed to remove the ether. Water and hydrochloric acid are added to make a final volume of 150 ml. of approximately 0.2 *M* hydrochloric acid and the solution is transferred to the first of ten numbered 500-ml. separating funnels. The succeeding funnels each contain 150 ml. of 0.2 *M* hydrochloric acid. To the first funnel is added 150 ml. of chloroform and the mixture is shaken to establish equilibrium. The chloroform layer is then passed on to the succeeding funnel. This procedure is continued until chloroform reaches the tenth funnel. The distribution of the components at this point may be calculated from their distribution coefficients with the aid

TABLE IX

THE CALCULATED DISTRIBUTIONS OF THE PIPSYL DERIVATIVES OF GLYCINE, ALANINE AND PROLINE BETWEEN CHLOROFORM AND DILUTE HYDROCHLORIC ACID, IN A TEN-PLATE COUNTER-CURRENT DISTRIBUTION

Compound	Phase	Per cent. of compound in each separatory funnel (funnel number) 1	2	3	4	5	6	7	8	9	10
Glycine	Aqueous	0.60	3.7	9.7	14.9	15.0	10.0	4.5	2.2	0.22	0.01
	Solvent	.40	2.4	6.4	9.9	9.9	6.7	3.0	0.86	.14	.01
Alanine	Aqueous	.00	0.00	0.00	0.06	0.34	1.4	3.6	5.9	6.0	2.7
	Solvent	.00	.00	.02	.22	1.4	5.6	14.3	23.7	24.1	10.7
Proline	Aqueous	.00	.00	.00	.00	0.00	0.00	0.00	0.1	0.7	2.4
	Solvent	.00	.00	.00	.00	.00	.00	.2	2.7	21.3	72.6

of the binomial expansion (Table IX). The experimental distributions have agreed well with those calculated.

By pooling the fractions as indicated below, one obtains pipsylglycine substantially free from the pipsyl derivatives of proline and alanine. The latter are combined in a single fraction from which pipsylproline may be removed by extraction with a mixture of chloroform and carbon tetrachloride.

Glycine Carrier.—The chloroform phase of funnels 2, 3 and 4 and the aqueous phase of funnels 3, 4 and 5 are combined and made alkaline (Table IX). The aqueous phase, which now contains the pipsylglycine is acidified and extracted with about 100 ml. of normal butanol. The butanol is washed with several volumes of water and then mixed with about 200 ml. of petroleum ether. The mixture is extracted twice with 10 ml. of 0.2 *M* NaOH and the alkaline extract is treated with charcoal and filtered into a centrifuge tube. Upon acidification with hydrochloric acid, pipsylglycine crystallizes out. The product is submitted to repeated alternate recrystallizations and charcoal treatments to constant specific activity.

Alanine Carrier.—The chloroform phase of funnel 8 and both phases of funnels 9 and 10 are combined, made alkaline and shaken. The aqueous layer is acidified with hydrochloric acid and extracted five times with a 60:40 mixture by volume of chloroform and carbon tetrachloride. The first three extracts are pooled for the proline estimation and the last two are discarded. The aqueous phase, which contains the pipsylalanine is extracted with butanol and further purified in the manner described for pipsylglycine.

Proline Carrier.—The three pipsylproline extracts referred to above are pooled and extracted twice with 10 ml. of 0.2 *M* NaOH. The alkaline solution is treated with dipipsylalanine and potassium chloride as described in an earlier section and is then further purified in the same way as the pipsylglycine.

The Preparation of Non-Isotopic Pipsyl Chloride.—This has been prepared in kilogram quantities for use in the preparation of carriers. *p*-Diazobenzene sulfonic acid is prepared[17] in quantities of about 100 g. at a time and treated with potassium iodide, while still wet, to yield *p*-iodophenyl sulfonic acid.[18]

The sodium salt of the latter is salted out with sodium chloride and dried. The acid chloride is prepared from this by addition of phosphorus pentachloride in phosphorus oxychloride. After reaction, the mixture is dissolved in benzene, washed with ice-water to remove phosphorus halides and dried over anhydrous sodium sulfate. Further purification by vacuum distillation below 150° is desirable. The acid chloride crystallizes on addition of excess of petroleum ether to the benzene solution in colorless crystals melting at 86° (literature, 86–87°).[19]

The Synthesis of Isotopic Pipsyl Chloride.—Quantities of a few hundred milligrams have been made at one time. For reasons of economy and sensitivity, the method of synthesis has been so modified as to incorporate as much as possible of the I^{131} into the product. The progress of the synthesis is controlled by radioactive counts on the intermediate products.

Isotopic Synthesis.—To the radioactive iodine,[20] in solution, in the form of iodide ion, is added sufficient potassium iodide to make a total of 25 mg. The solution is adjusted to a *p*H above 7 and evaporated to a volume of less than 0.5 ml. The sample is transferred quantitatively, with the aid of several small portions of water, to a Pyrex test-tube and the volume is reduced to about 0.2 ml. A few small crystals of sodium sulfite are added. After cooling, an equal volume of concentrated hydrochloric acid is added followed by 25 mg. of diazobenzenesulfonic acid. After the initial evolution of nitrogen the sample is warmed to complete the reaction. The tube is cooled again and another 15 mg. each of diazobenzenesulfonic acid and potassium iodide are added and allowed to react as before. The reaction mixture is then saturated with sodium chloride and cooled, whereupon the sodium salt of *p*-iodophenylsulfonic acid crystallizes out. The crystals are centrifuged down and washed with brine. The combined mother liquor and washings are warmed and about 40 mg. of non-isotopic sodium iodophenylsulfonate, in solution in a small volume of water, is added. Upon cooling, the added sodium salt crystallizes out carrying down residual isotopic analog. This is also centrifuged down and washed with brine and the procedure repeated. The separate batches of sodium salt are dried and then each is dissolved in about one ml. of phosphorus oxychloride containing a substantial excess of phosphorus pentachloride. The batches are pooled and the mixture is heated gently to ensure reaction. The reaction mixture is transferred to a separatory funnel containing ice-water and about 50 ml. of benzene. Small portions of benzene are used to facilitate the transfer from the test tubes to the funnel. At this point, approximately 200 mg. of pure non-isotopic pipsyl chloride is added. The benzene layer is washed several times with cold water to remove excess phosphorus halides. The rate of hydrolysis of pipsyl chloride is negligible, under these conditions, as is indicated by the absence of any appreciable amounts of radioactivity in the wash water. The benzene solution is dried over anhydrous sodium sulfate and the drying agent is washed with benzene until it is substantially free of radioactivity.

The combined benzene solution is evaporated to a small volume and transferred to a cold finger micro-distillation apparatus. The benzene is evaporated in a stream of air and the pipsyl chloride is distilled onto the cold finger at 150° at a few mm. of mercury pressure. The distilled material is washed into a vessel with a minimum of benzene. At this point more non-isotopic pipsyl chloride

(17) C. Weygand, "Organic Preparations," Interscience Publishers, Inc., New York, N. Y., 1945, p. 109.

(18) C. Weygand, *ibid.*, p. 112.

(19) W. Lenz, *Ber.*, **10**, 1136 (1877).

(20) The radioactive iodine used in the early part of the work was obtained through the kindness of Dr. Robley D. Evans of the Department of Physics at the Massachusetts Institute of Technology. The I^{131} used more recently in this investigation was supplied by the Clinton Laboratories on allocation from the U. S. Atomic Energy Commission. An irradiation unit containing approximately 100 millicuries of I^{131} was used for each isotopic synthesis. The I^{131} was separated from the tellurium by a procedure developed in this Laboratory (M. Levy, A. S. Keston and S. Udenfriend, THIS JOURNAL, **70**, 2289 (1948)). Isotopic pipsyl chloride was prepared at approximately six-week intervals.

may be added to make a desired specific activity. The pipsyl chloride must be completely dissolved and thoroughly mixed to ensure isotopic homogeneity. Recrystallization may be achieved by adding petroleum ether to the benzene and concentrating the solution. The small amount of residual solvent is then drawn off with a capillary pipet and the crystals are dried at 70° for about one hour. The reagent has been successfully used even when the last recrystallization was omitted.

The Synthesis of Pipsyl Amino Acids for Use as Carriers.—In the case of the monobasic amino acids, an excess of amino acid, in aqueous solution, is heated with pipsyl chloride in the presence of enough sodium bicarbonate to neutralize the acids formed during the reaction. Efficient stirring must be provided and the temperature maintained near boiling until the pipsyl chloride has all reacted. Potassium chloride is then added to precipitate some dipipsyl impurities which have very insoluble potassium salts and the reaction mixture is treated with activated charcoal and filtered. Crystals of the derivative generally appear after acidification and cooling of the filtrate. If they do not, the compound may be extracted into ether or ethyl acetate and then returned to a small volume of alkali. Upon acidification, the crystalline derivative will then appear. The derivatives may be recrystallized either from hot water or from a mixture of acetone and water. The crystals are dried at 105°. The properties of a number of derivatives are given in Table III.

Discussion

Hevesy and Hobbie[21] in 1932 were the first to use isotopes to overcome the difficulties of analytical methods which required the quantitative isolation of a pure sample from a mixture. They developed a microanalytical method for the analysis of lead in ores which involved adding a measured trace amount of radium D, which is isotopic with lead, to the dissolved ore and then separating a pure sample of lead from the mixture by standard chemical procedures. The total amount of lead present in the sample at the start of the analysis was then found by dividing the amount of lead isolated by the fraction of radioactivity recovered. The use of isotopically prepared organic compounds, in a manner similar to radium D in the analysis of lead, has come to be known as the isotope dilution method.[22,23,24]

The analysis of radioactive constituents in a mixture by carrier techniques has been a commonplace for a long time in the fields of radioactivity and its biological applications. The simplest statement of the new method described here is that the compounds of interest are converted into radioactive substances by direct chemical binding with a radioactive reagent.

The isotopic derivative method, presented here, has much higher sensitivity than the isotope dilution technique, being operable below the microgram level, and has the inherent specificity and accuracy characteristics of isotopic methods. One isotopic reagent suffices for the determination of many compounds. It is also possible to check analytical values with many reagents of different types. The use of large amounts of carrier makes possible rigorous isotopic purification. The use of large amounts of racemic carrier, moreover, enables one to estimate simultaneously both forms of an optically active compound, eliminating any errors that might arise due to racemization during preparation. The method may be made optically specific by using the appropriate enantiomorph as the carrier. Furthermore, the estimation of naturally occurring compounds whose syntheses present difficulties may be accomplished, since it is usually easier to prepare a derivative as a carrier than to synthesize the compound itself in isotopic form.

The significance of co-precipitation to the problem of the purification of isotopic compounds has been emphasized above. Co-precipitation is a phenomenon familiar to inorganic chemists.[25] It is evidently common in mixtures of related organic compounds also. We suggest that more attention should be paid to it in all tracer studies in which the tracer is isolated from a complex mixture and then purified.

The isotopic derivative method may also be applied where quantitative conversion to a derivative is not achieved. The addition of a trace amount of the compound to be determined, labelled with a second isotope which can be distinguished from that used in the reagent, enables one to calculate and correct for the extent of conversion to the derivative.

Pipsyl chloride has been applied to the determination of peptides in partial hydrolysates of proteins[26] and pipsyl chloride containing S^{35} as the isotopic label has been made and used as an indicator in the application of the procedure to paper chromatography.[5] The application of the procedure to the systematic analysis of proteins, using both the carrier technique and paper chromatography, is now in progress.

Summary

A new principle for the application of isotopes to the determination of organic compounds is introduced and its application to the determination of amino acids is presented.

A mixture is treated with an isotopic reagent to form quantitatively an isotopic derivative of the desired amino acid. A large excess of the unlabelled derivative is added as carrier and purified to constant isotope concentration. Knowing the specific radioactivity of the purified carrier and of a suitably prepared standard and also the fraction of carrier remaining after purification, one can calculate the quantity of amino acid present in the original mixture.

As reagent *p*-iodophenyl sulfonyl chloride (pipsyl chloride) containing I^{131} was used.

(21) G. Hevesy and R. Hobbie, *Z. anal. Chem.*, **88**, 1 (1932).

(22) R. Schoenheimer, S. Ratner and D. Rittenberg, *J. Biol. Chem.*, **130**, 703 (1939).

(23) H. H. Ussing, *Nature*, **144**, 977 (1939).

(24) D. Rittenberg and G. L. Foster, *J. Biol. Chem.*, **133**, 737 (1940).

(25) I. M. Kolthoff and V. A. Stenger, "Volumetric Anal.," Vol. I, 2nd ed., 1942, pp. 182–205.

(26) M. Levy and E. Slobodiansky, *Federation Proc.*, **7**, 168 (1948).

The method has extremely high sensitivity, being operable below the microgram level, and has the inherent specificity and accuracy characteristic of isotope techniques.

Recoveries of added amounts of glycine, alanine and proline from a mixture of twelve synthetic amino acids are reported to demonstrate the accuracy and specificity of the analytical procedure.

Values are reported for the glycine, alanine and proline contents of the crystalline proteins β-lactoglobulin, human hemoglobin, aldolase and phosphoglyceraldehyde dehydrogenase.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, TENNESSEE AGRICULTURAL & INDUSTRIAL STATE COLLEGE]

Preparation and Reactions of Several Chlorine Substituted Phenoxyacetyl Chlorides

BY CARL M. HILL, MYRON B. TOWNS AND GILBERT SENTER

Chemical investigation involving study of the several phenoxy acetic acids and their derivatives has been chiefly concerned with their insecticidal and herbicidal activity. Of the six phenoxy substituted acetic acids (phenoxy, 2-chlorophenoxy, 4-chlorophenoxy, 2,4-dichlorophenoxy, 2,4,5-trichlorophenoxy and 2,4,6-trichlorophenoxy) the derivatives of the two trichloro acids described in this paper are not reported in the literature. It was of interest, therefore, to investigate the preparation and properties of these derivatives and to establish their identification.

Inasmuch as the phenyl esters (except phenyl phenoxyacetate) of the six acids mentioned above are not described in the literature, these were prepared and identified.

Experimental

Acids.—The 2,4,5- and 2,4,6-trichlorophenoxyacetic acids were obtained from Dow Chemical Company and were purified by recrystallization from chloroform; 2,4,5-trichlorophenoxyacetic acid, m. p. 152–153° (uncor.) (lit.,[1] m. p. 154–55°), neut. equiv. calcd. for $C_8H_5O_3Cl_3$ 255.5, found, 257.7; 2,4,6-trichlorophenoxyacetic acid, m. p. 177–178° (uncor.), (lit.,[1,2] m. p., 190°, 177°), neut. equiv. found 256.0.

2,4,5-Trichlorophenoxyacetyl Chloride.—Into a round-bottomed flask equipped with a condenser set for reflux was placed 64 g. of 2,4,5-trichlorophenoxyacetic acid. One hundred and nineteen grams of purified thionyl chloride were added dropwise to the acid over a period of twenty-four hours. At the end of this time, the reaction mixture was refluxed on a steam-bath for two hours. The excess thionyl chloride was removed by distillation at atmospheric pressure. Distillation of the residue from a modified Claisen flask, under reduced pressure, gave 54.8 g. (80.0%) of 2,4,5-trichlorophenoxyacetyl chloride; b. p. 165–167° (6 mm.). This product was a waxy, white solid with a cocoanut-oil odor; m. p., 78–79° (uncor.).

Anal. Calcd. for $C_8H_4O_2Cl_4$: ionizable Cl, 12.94. Found: Cl, 12.46.

2,4,6-Trichlorophenoxyacetyl Chloride.—This compound was prepared by the method described for 2,4,5-trichlorophenoxyacetyl chloride using 64 g. of 2,4,6-trichlorophenoxyacetic acid; yield 61.6 g. (90.0%). This product was a waxy, white solid; b. p. 147–150° (5 mm.), m. p., 55–56° (uncor.).

Anal. Calcd. for $C_8H_4O_2Cl_4$: ionizable Cl, 12.94. Found: Cl, 13.03.

(1) Synerholm and Zimmerman, *Contrib. Boyce Thompson Institute*, **14**, 91 (1945); *Chem. Abst.*, **39**, 1474 (1946).

(2) Haskelberg, *J. Org. Chem.*, **12**, 426 (1947).

Both chlorides were analyzed for ionizable chlorine according to the following procedure: Fifty ml. of ethanol, 15 ml. of 2 *M* potassium hydroxide and 0.5 g. of the acid chloride were refluxed for twenty minutes in an erlenmeyer flask attached to a water-cooled condenser. The solution was allowed to cool, after which 50 ml. of distilled water was added through the head of the condenser. The solution was transferred to a 400-ml. beaker and the washings from the flask were added. After acidifying the solution with 1:1 nitric acid, the chlorine was precipitated with 0.5 *M* silver nitrate. The solution was then heated, with stirring, and the precipitated silver chloride filtered. The precipitate was washed on filter with 1:1 nitric acid followed by ethanol, dried and weighed as silver chloride.

Amides

2,4,5-Trichlorophenoxyacetamide.—To 0.5 g. of 2,4,5-trichlorophenoxyacetyl chloride was added a slight excess of concentrated ammonia and the mixture heated over a water-bath until precipitation of the amide was complete. The crude amide was filtered and recrystallized from dilute ethanol. The pure product was a white solid; m. p. 173–174° (uncor.), yield 0.50 g. (97.5%).

Anal. Calcd. for $C_8H_6O_2Cl_3N$: N, 5.50. Found: N, 5.81.

2,4,6-Trichlorophenoxyacetamide.—This amide was prepared in the manner described for the 2,4,5-trichlorophenoxyacetamide, starting with 0.5 g. of 2,4,6-trichlorophenoxyacetyl chloride. This product was a white crystalline solid; m. p. 195–196° (uncor.), yield 0.50 g. (98.0%).

Anal. Calcd. for $C_8H_6O_2Cl_3N$: N, 5.50. Found: N, 5.59.

Ethyl Esters

Ethyl 2,4,5-Trichlorophenoxyacetate.—To 5 g. of 2,4,5-trichlorophenoxyacetyl chloride was added a slight excess of 95% ethanol. The mixture was heated under reflux for two hours, allowed to cool, and washed with a saturated solution of sodium bicarbonate and then with water. The crude ester was extracted with diethyl ether and the ethereal solution dried over anhydrous calcium chloride. After removal of the ether, the crude ester was recrystallized from dilute ethanol. This product was a white crystalline solid; m. p. 63–64° (uncor.), yield 5.1 g. (89%), sap. equiv. calcd. for $C_{10}H_9O_3Cl_3$ 283.6, found 281.0.

Ethyl-2,4,6-Trichlorophenoxyacetate.—Five grams of 2,4,6-trichlorophenoxyacetyl chloride was used; yield 2.8 g. (85%). This ester was a white crystalline solid; m. p. 33–34° (uncor.), sapn. equiv. found, 280.0.

Anilides

2,4,5-Trichlorophenoxyacetanilide.—A solution of 5 g. of 2,4,5-trichlorophenoxyacetyl chloride and 15 ml. of chloroform was placed in an erlenmeyer flask. To this was added a slight excess of pure aniline. The reaction mixture was heated under reflux for thirty minutes and then allowed to cool. The crude anilide was filtered off