THE USE OF RADIOISOTOPICALLY LABELED ANALYTICAL REAGENTS IN ORGANIC CHEMISTRY

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Contents

I. Introduction

This review covers the major applications of labeled reagents in organic chemistry appearing in the literature up to the end of 1969. As the title suggests, the examples considered involve quantitative or near-quantitative reaction of an isotopically labeled reagent with a particular functional group contained in the molecule to be determined. Consequently, the review is classified into sections dealing with the determination of particular functional groups. The use of isotopes merely as tracers or indicators, of which there are a countless number of examples in the literature,[4-9] is not considered.

The whole field of analytical radiochemistry is well documented and there are several excellent general texts to which the reader is referred.[10-13] The actual synthesis of labeled reagents and compounds is not generally discussed;[10-17] neither is the method of measurement of ionizing radiation described in detail since there are several review articles dealing with liquid scintillation counting,[18-21] gas counting,[22-25] and autoradiography[26-29] specifically, and measurement generally.[30-32]

II. Analytical Procedures

A. INTRODUCTION

There are basically two, related procedures relevant to this article, both involving formation of an isotopically labeled derivative of the material under investigation. In many cases, quantitative reaction with the reagent is essential but one of the advantages of radioisotope methods is that this requirement

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may be relaxed if a labeled form of the substance being determined is available (*e.g.*, see ref 37). For larger samples a quantitative recovery of the labeled material is not usually required, and it is only necessary to isolate sufficient of the purified derivative to enable its radioactivity to be accurately determined. From a knowledge of the weight of sample taken and the specific activity of the labeling reagent used, it is then possible to calculate the number of functional groups per unit mass of substrate. It is a prerequisite condition, however, that the labeled material must be completely free from unreacted reagent and labeled impurities.

When dealing with samples at the milligram level and below, where a knowledge of the total quantity of unknown material is desired, losses will inevitably occur during the isolation and purification stages. Application of a double isotope derivative analysis technique, where one isotope gives a measure of the amount of unknown, while the second allows correction for any losses during the analytical procedure, can overcome these problems, since such a method requires only the determination of radioactivity. Thus, the criterion of chemical purity is replaced by one of radiochemical purity. The only labeled compound in the final assay sample must be the one of interest, although other unlabeled compounds may be present, provided that they do not interfere with the counting procedure. For this reason it is possible to add milligram amounts of carrier to nanogram amounts of sample of doubly labeled derivative in order to facilitate the radiochemical purification, without altering the accuracy or sensitivity of the method in any way.

The sensitivity of double isotope techniques depends on the specific activity of the labeled reagent available, and recent advances in the preparation, purification and storage of these labeled materials have made them readily available at high specific activity, thus increasing the sensitivity of the analysis to nanogram levels. The study of hormones is a field in which these techniques have found particular application.

Pioneers in the field of isotope derivative analysis were Keston, Udenfriend, and Cannan,[33] who in 1946 used [^{131}I]-*p*-iodophenylsulfonyl chloride (pipsyl chloride) to react quantitatively with an unknown mixture of amino acids. A known excess of each of the unlabeled pipsyl derivatives was then added to the mixture of labeled derivatives and pure specimens were isolated. The activity of these samples was determined by counting and the chemical purity by a conventional analytical technique.

This procedure, even after further development,[34] still involved isolation of a pure sample large enough for conventional nonisotopic analysis. An improvement of the method[35,36] eliminated this; after preparation of the ^{131}I-labeled pipsyl derivatives of all the amino acids in the unknown mixture, a known quantity of ^{35}S-labeled derivative of the amino acid under assay was added. Then unknown amounts of unlabeled carrier pipsyl derivative could be added during the purification and the weight of amino acid in the original sample was determined from the ratio of the ^{35}S/^{131}I counts in a radiochemically pure specimen of the derivative.

This double isotope derivative technique was further refined by Keston and Lospalluto[37] in 1951. Known samples of the amino acids labeled with ^{14}C were added before esterification and a similar determination was carried out. This technique had the added advantage of compensating for any losses during esterification, and still enabled the amino acids to be determined from the ^{14}C/^{131}I ratio.

Further developments of these methods have led to a large number of very refined analytical techniques for the determination of amino acids, proteins, peptides, and steroids in biological systems at levels down to 10^{-11} g. The most significant advances were the introduction of tritium-labeled compounds and reagents with specific activities in excess of 100 mCi/mmol, the development of chromatographic separation techniques for purification of derivatives, and the availability of liquid scintillation spectrometers for counting weak β emitters, including simultaneous determination of two isotopes.

The two basic methods of isotope derivative analysis and double isotope derivative analysis are discussed briefly below, but for a fuller derivation of the general mathematical principles the reader is referred to the general texts mentioned earlier and to an excellent article on the isotope derivative method.[38] The general principles of isotope dilution assays were also discussed in a much earlier paper.[39]

B. ISOTOPE DILUTION ANALYSIS

Although this method of analysis does not strictly fall within the scope of this article, the principle of isotope dilution is very important to an understanding of derivative techniques. The basic principle depends on the fact that, if a radioactive tracer is mixed with the corresponding unlabeled compound, the specific activity of the active compound will be reduced by a factor relative to the proportions of the two materials. If the reduction in specific activity can be measured, the amount of diluting material added can be calculated.

Thus, to analyze for the unknown quantity m_i, moles of an inactive compound, A, in a mixture of compounds A + B + C + D + . . ., a radioactive form of the same compound, A*, is required. A known quantity, m, moles of the compound A* whose mole specific activity, S_0, has been measured, is added to the mixture. The total activity, a, added is given by

$$a = mS_0 \quad (1)$$

and after dilution with unknown

$$a = (m + m_i)S \quad (2)$$

where S is the mole specific activity of the diluted compound. From eq 1 and 2

$$m_i = m[(S_0/S) - 1] \quad (3)$$

The great advantage of this method is that it is not necessary to separate the whole of $(m + m_i)$ to measure S. Provided that the labeled compound and the inactive component are in the same chemical form and have been thoroughly mixed, then the specific activity is independent of the amount of material used to measure it. As long as some of the component can be separated in a pure state, the amount of inactive material in the sample can be determined.

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C. ISOTOPE DERIVATIVE ANALYSIS

In this procedure the unknown compound is made to react quantitatively with a radioactive reagent of known specific activity, and the radioactive product is separated and purified either directly or after dilution with pure inactive derivative. The latter technique is often referred to as "isotope derivative dilution analysis" (see below). The amount of activity in the pure product indicates the amount of radioactive reagent it contains, and, as the stoichiometry of the reaction between reagent and compound is known, the amount of compound present can be calculated.

If S_r is the activity per equivalent of the reagent (taking into account the stoichiometry of the reaction) and a the measured total activity of the quantitatively isolated derivative, then

$$m_i = a/S_r \tag{4}$$

Simple isotope derivative analysis, without dilution, is rarely used since it is almost impossible to isolate quantitatively the radiochemically pure material. The process can be simplified as mentioned above, by isotope derivative dilution. This is a special case of the more general "reverse isotope dilution technique" whereby a radioactive product in a radioactive mixture can be analyzed by addition of inactive carrier material followed by isolation and purification. The technique is often used to establish radiochemical purity of a labeled compound (*e.g.*, as required in section E below). In the present case, radioactive derivative formation is followed by addition of a large known quantity m_{id}, moles of pure inactive derivative (carrier). Isolation of a quantity m_d, moles of pure derivative, is then carried out and its activity, a_d, measured. The specific activity, S_d, of this material is given by

$$S_d = a_d/m_d \tag{5}$$

And since as in eq 1 and 2 the total activity remains constant

$$a = m_i S_r = (m_i + m_{id})S_d \tag{6}$$

therefore

$$m_i = m_{id}S_d/(S_r - S_d) \tag{7}$$

or alternatively

$$m_i = (m_i + m_{id})S_d/Sr \tag{8}$$

and in many practical cases, where $m_{id} \gg m_i$, eq 8 reduces to

$$m_i = m_{id}S_d/S_r \tag{9}$$

It must be stressed that the activities a and a_d are not the same; a refers to the total activity of the isolated, undiluted derivative, if this were ever carried out, whereas a_d is the measured activity of a portion of the diluted sample.

D. DOUBLE ISOTOPE DERIVATIVE ANALYSIS

The sensitivity of the isotopic technique of analysis may be significantly increased by the combination of isotope dilution and isotope derivative methods. In this method two different radioactive isotopes are used, the first in a straightforward isotope derivative analysis, and the second in an isotope dilution as a check on the losses during purification of the derivative. Thus, the ratio of counts in the final sample due to the second isotope to the total counts of that isotope added initially will provide a correction factor for losses of the first isotope. The second isotope can be introduced either before or after the derivative has been formed, that is, either by the addition of a small amount of the labeled compound to be analyzed or of labeled derivative. The latter can be labeled either in the reagent residue or in the compound. These variations are discussed below.

1. Labeled Compound Method

When the compound to be determined is available in a labeled form, a known quantity, m, of labeled compound of known specific activity, S_0, is added to the mixture as described in section B. The compound is extracted and partially purified before the derivative is formed using a reagent labeled with the second isotope and of activity per equivalent, S_r. The derivative is isolated, probably by chromatography, and then counts due to the two isotopes are determined. If a_1 equals the activity due to the first isotope, the factor to correct for losses during purification will be

$$mS_0/a_1 \tag{10}$$

If a_2 is the activity due to the second isotope in the isolated derivative, the total activity due to this isotope after correction for losses during isolation is

$$a = a_2 mS_0/a_1 \tag{11}$$

and as before

$$m_i = a/S_r \tag{12}$$

Therefore

$$m_i = a_2 mS_0/a_1 S_r \tag{13}$$

2. Labeled Derivative Method

The unknown is caused to react quantitatively with reagent of activity per equivalent, S_r, as before, but before purification a known quantity, m_{ad}, of indicator derivative of specific activity S_{ad} is added to the crude reaction mixture. This second indicator derivative is identical chemically with that formed in the reaction mixture but is labeled with a different isotope.

If a_1 is the measured activity due to indicator derivative and a_2 that due to the reagent isotope which reacted with the unknown, then an expression for m_i can be derived.

$$m_i = a_2 m_{ad}S_{ad}/a_1 S_r \tag{14}$$

E. LABELED REAGENTS

The required properties of a labeled reagent for use in isotope derivative analysis may be summarized as follows: it should be available at a high specific activity (preferably >100 mCi/mmol), easy to store, handle, and dilute, and in addition react quantitatively with the compound in a crude form. The radiochemical purity of the reagent must be established beyond doubt, preferably by isotope dilution as well as by other conventional techniques. The isotope in the reagent should be such that a tracer compound or derivative can be easily prepared at high specific activity using a second isotope. The reagent should be selective for the compound under assay and not react with metabolites and/or related compounds in the case of biological systems. The derivative obtained should be readily extractable from the reaction mixture and easily purified. Finally, the reagent isotope used along with a second isotope must be such that the counting procedure is as simple

and yet as accurate as possible and the reagent should give a low blank value.

Many of the examples set out in the following sections refer to sensitivities of the order of a few milligrams or micrograms. It is worth reemphasizing that most methods can be developed to give detection limits of a few nanograms as and when labeled reagents and indicators become available at higher specific activities than were available to the original workers.

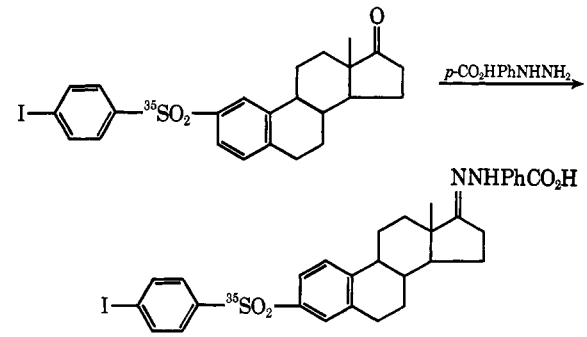
III. Analysis of Hydroxyl Compounds

By far the greatest use of labeled reagents for determination of hydroxylic compounds has been in the analysis of steroids, and for a general reference to the qualitative and quantitative analysis of these compounds the reader is referred to a recent book.[40]

Several labeled reagents have been used in the double isotope derivative procedure of Keston[33-37] for the submicrogram determination of steroid hormones. The first workers to apply this technique used the original [^{131}I]-*p*-iodophenylsulfonyl (pipsyl) chloride for the analysis of corticosteroids.[41] This method, which is described in greater detail in the section dealing with amino group determination, proved unsatisfactory owing to low yields of the pipsyl esters and unwanted side reactions, although the reagent was satisfactory for the esterification of estrone and 17β-estradiol.[42-44] Svendsen[43] used the more stable ^{35}S isotope in the derivative-forming reagent and the ^{131}I species as the indicator, thus greatly enhancing the sensitivity of the method. The steroid in question was quantitatively separated from plasma by extraction and purified by partition chromatography before esterification with [^{35}S]pipsyl chloride. After addition of [^{131}I]pipsyl indicator, final purification was effected by repeated paper chromatography, the bands being located by autoradiography and measured by radiochromatogram scanning. The sensitivity of this method was given as 2 ng, and recoveries of estrone and 17-β-estradiol were 65 ± 9 and 64 ± 6%, respectively. By comparison, a single isotope derivative technique claimed a lower limit of detection of 0.3 μg of estrogen.[42]

The same procedure, but with higher specific activity [^{35}S]-pipsyl chloride (100 instead of 60 mCi/mmol) and a rather more involved chromatographic purification, increased the sensitivity to 1 ng of estrone and 17β-estradiol.[44] After the second paper chromatogram, the estradiol ester was applied to the origin of the third chromatogram and oxidized with aqueous chromic acid to estrone before subsequent development. The estrone ester was converted to *p*-carboxyphenylhydrazone on the portion of paper cut from the second chromatogram by addition of *p*-carboxyphenylhydrazine hydrochloride solution (eq 15). The hydrazine was then eluted onto the starting line of a third paper and developed.

This procedure has since been applied to the determination of the two estrogens and estradiol precursors in plasma[40,45] with a similar sensitivity. A most important modification made by the later workers was the use of ^{125}I, a very weak γ emitter,



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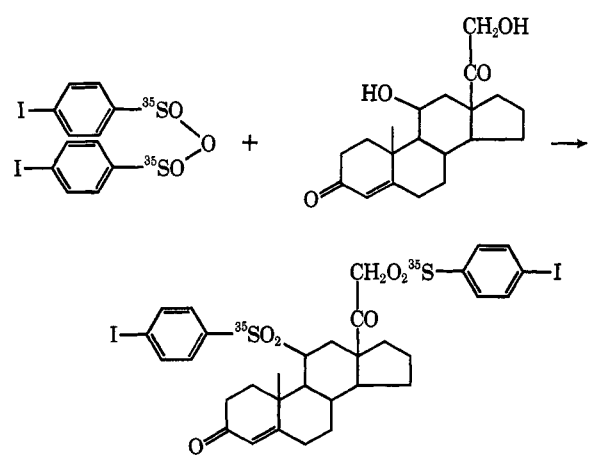
instead of ^{131}I as the isotope for labeling indicator pipsyl derivatives. This enabled them to count β- and γ-emitting isotopes simultaneously. A methane flow counter with a thin plastic window was used to determine ^{35}S at 10% efficiency with a 2% breakthrough of ^{125}I in conjunction with a crystal-scintillation detector which would determine the ^{125}I with only a 0.007% breakthrough of ^{35}S. The big advantage of ^{125}I over ^{131}I was that it is a very much weaker γ emitter, and therefore fewer precautions were necessary against radiation hazards. ^{125}I also has a much longer half-life (60 days as opposed to 8.04 days); therefore less restandardization was required to correct for decay.

Recently determination of estradiol and estrone in avian plasma has been reported using triply labeled derivatives.[46] [^{3}H]-17β-Hydroxyestradiol was added to the plasma before partial purification of the phenolic steroids, which were then treated with [^{35}S]pipsyl chloride of high specific activity. Standard [^{131}I]pipsyl derivatives were added and the esters purified by paper chromatography. The estrogens were determined by comparison of the ratios of ^{3}H to ^{35}S and ^{131}I to ^{35}S in the purified esters with the corresponding ratios of appropriate standards. Recoveries of estrone and estradiol after hydrolysis of the plasma were 70–85 and 72–84%, respectively, and after hydrolysis and preliminary purification, 38–53 and 39–51%, respectively. The method was suitable for samples containing up to 500 ng of estradiol, with a sensitivity of 2.1 ng for this and 3.0 ng for estrone.

After initial unsuccessful attempts to esterify corticosteroids with pipsyl chloride,[41] subsequent use of [^{35}S]-*p*-iodophenylsulfonyl anhydride (pipsan) for cortisol, corticosterone, and 11-deoxycorticosterone was shown to be satisfactory.[47]



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With [^{131}I]pipsyl derivatives as indicators and paper chromatographic purification, it was possible to measure 0.2–10 μg of steroid in 5 ml of human and dog peripheral plasma, with a standard deviation of ±6%. Later the sensitivity of the method was improved by using [^{125}I]pipsyl derivatives as indicators in place of the ^{131}I species.[40] A 56% recovery was achieved for cortisol, with a standard deviation of ±0.03 μg. Figures were also quoted for cortisone, deoxycortisol, and heparinized plasma.

Pipsan has also been used to determine 17-hydroxycorticosterone in human plasma, but apparently without an indicator derivative, although a technical error of only ±1.1 was claimed.[48] Pipsan and [^{131}I]pipsyl indicator were used for the determination of aldosterone in plasma from heparinized blood.[49] Reverse phase paper chromatography and the chromic acid oxidation treatment referred to earlier were used, and a level of estimation of 1–10 ng coupled with a recovery of 80% was claimed.

A considerably modified version of this procedure was used for determination of picogram quantities of aldosterone in plasma.[40] In this case the indicator was [^{3}H]aldosterone of very high specific activity (30–50 Ci/mmol) which was added to the plasma sample before extraction and derivative formation and which could thus give an indication of all losses. The labeled reagent was high specific activity [^{35}S]"Tosan" (*p*-toluenesulfonyl anhydride) (150 mCi/mmol) as opposed to [^{35}S]-pipsan or [^{35}S]pipsyl chloride. Both paper and thin layer chromatographic techniques were used for purification of the derivative. After a second chromatogram, the aldosterone tosyl ester was oxidized with chromic acid, washed, and subjected to tlc, a second time (third chromatogram). After the third chromatogram it was converted to its 2,4-dinitrophenylhydrazone and purified by either reverse phase or thin layer chromatography. In either case 2,5-diphenyloxazole (PPO) was added to the stationary phase of the thin layer plate to facilitate detection of the derivative under uv light. The limit of detection by this method was claimed to be 50 pg.

Although the first reagents used for the double isotope derivative analysis of steroids were the labeled sulfonylating reagents,[48–49] the most widely used reagent for the estimation of submicrogram quantities of these hydroxylic compounds is undoubtedly [^{3}H]- or [^{14}C]acetic anhydride. In utilizing such a reagent it is important that one knows which groupings are acetylatable under the conditions used, the degree with which they react with the labeled reagent, and the conditions necessary for a single unknown to give rise to a single product. The reactivity of the various acetylatable groupings of the steroids was studied using [1-^{14}C]acetic anhydride.[50] Appreciable differences were found depending on substituent position, the relative order to reactivity being given as 3-OH (phenolic) > 21-OH > 3β-OH > 6β-OH > 21α-OH > 20β-OH > 16α-OH > *sec*-17β-OH > *sec*-17α-OH. Concentration of steroid over the range of 0.6–100 μg per 0.1 ml of acetylating agent did not seem to influence the relative degree of acetylation, and for the composition of the acetylating mixture, a ratio of 1:5 acetic anhydride:pyridine appeared to be the most satisfactory for a wide variety of steroids to achieve a single product and complete reaction.

As early as 1953 [^{3}H]acetic anhydride was used for double isotope derivative analysis of aldosterone and hydrocortisone in human peripheral blood.[51,52] [^{14}C]Aldosterone or [^{14}C]-hydrocortisone acetate was added as indicator and the derivative purified by partition column chromatography. Although the method worked satisfactorily for hydrocortisone, with a sensitivity of 2.5 ± 0.5 μg per 100 ml of blood, it was not really successful with aldosterone for several reasons. The aldosterone diacetate was not radiochemically pure even after three elutions through a partition column chromatograph, and the specific activity of the [^{3}H]acetic anhydride was too low, as the peripheral plasma contained only extremely small quantities of aldosterone.

Around the same time acetylatable steroids were investigated generally using [^{14}C]acetic anhydride as the derivative-forming reagent.[53–55] Berliner[53] initially did not use an isotopic indicator, but after paper chromatography he was able to quantitatively recover 0.1 μg. Later[54] he described a general method applicable to a wide number of hydroxy steroids in which unlabeled steroid acetate was added as a carrier and chromic acid oxidation used in identification on the paper chromatograms. He actually measured 0.86 μg of cortisol in 10 ml of plasma, but with other sterols the method was qualitative only.

It was not until 1960, however, that the double isotope method was fully developed by Kliman and Peterson[56] for the determination of aldosterone in urine and plasma. With high specific activity [^{3}H]acetic anhydride (100 mCi/mmol), [^{14}C]-aldosterone diacetate as indicator, and the chromic acid oxidation between the second and third paper chromatograms, 0.1 μg of aldosterone in 5–30 ml of human urine or 2–3 ml of dog adrenal vein plasma was estimated with an overall recovery of the final purified product of 10% (*i.e.*, 0.01 μg).

A slightly different derivative ratio analysis for the measurement of steroids and other compounds with specific functional groups was described, using radioassay by gas–liquid chromatography.[57] An unlabeled internal standard possessing the same functional group as that being determined was added to the substance to be measured. The mixture was then acetylated with [1-^{14}C]acetic anhydride following which the derivatives were separated by gas–liquid chromatography. Detection and measurement of ^{14}C in the effluent of the chromatograph were performed either continuously during the analysis or by fractionating the effluent and then assaying each fraction individually. The quantity of unknown acetylated derivative was related to the quantity of standard in the same ratio as the radioactivity of the unknown was related to the radioactivity in the standard. The sensitivity obviously depended on the specific activity of the [1-^{14}C]acetic anhydride but the method has been used successfully for the determination of nanogram quantities of steroids.

Since all the later double isotope derivative methods using labeled acetic anhydride were either developments of the Kliman and Peterson method or very similar in procedure

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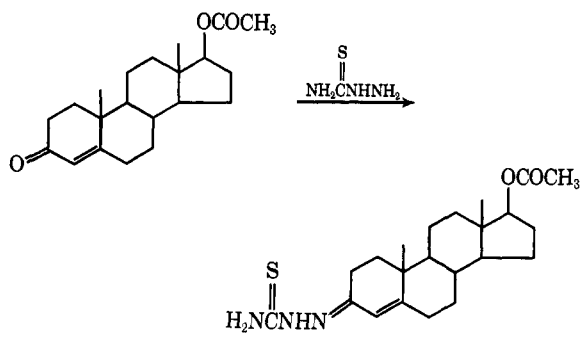
and detail, only the more important of them will be discussed with respect to particular groups of steroids.

A. CORTICOSTEROID

Paper chromatography was used for cortisol, with inactive cortisol acetate as carrier and ^{14}C-labeled steroid as indicator.[58] Chromic acid oxidation to cortisone was carried out between the second and third chromatogram, and 60–65% recovery was claimed up to the acetylation stage, with an overall recovery of 40–60%. An extra chromatography step prior to oxidation (four in all) was used for cortisol and achieved a sensitivity of 0.1 μg using ^{14}C-steroid acetate as indicator but without the addition of carrier.[59–61]

B. ANDROGENS

For testosterone, the thiosemicarbazide derivative was formed after running the third of five different paper chromatograms, after which time the final mean recovery was 17%[62] (eq 17). The sensitivity was given as 0.05 μg per 100 ml of plasma

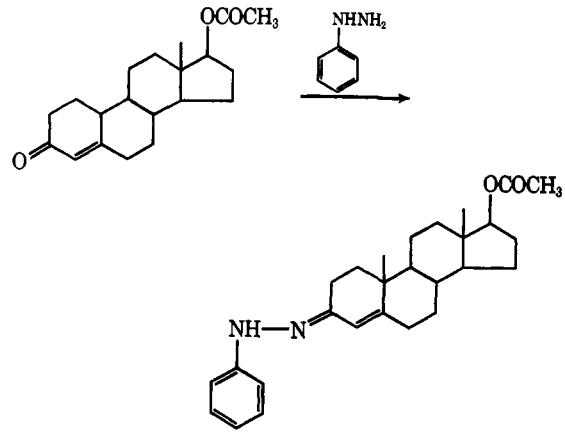


(17)

and the accuracy as ±10% at the 0.75-μg level and ±16% at the 0.14-μg level. Later, using two-dimensional tlc in addition to paper chromatography, but without formation of the thiosemicarbazide, much greater accuracy (±1.8%) with a similar sensitivity to the previous method was achieved.[62,63]

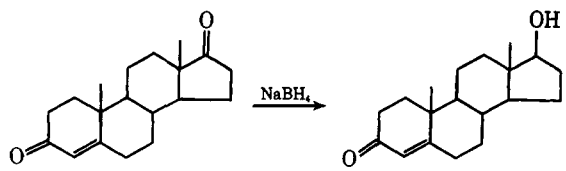
Testosterone, dehydroepiandrosterone, androsterone, and eticholanolone have been determined simultaneously in plasma.[64] Both tlc and paper chromatography were used for preliminary purification of the steroids and, after acetylation, the 1,1-dimethylhydrazones were prepared and separated by gas–liquid chromatography. The sensitivity and accuracy appeared to be comparable with that of other workers.

Partition column chromatography has also been used for purification of testosterone prior to acetylation, [4-^{14}C]-testosterone being added to the plasma as indicator before initial extraction.[65] The acetylated steroid was separated by two-dimensional tlc and partition column chromatography; the phenylhydrazone of testosterone acetate was then formed before the final chromatography to assist separation (eq 18).



(18)

A sensitive method for the simultaneous determination of testosterone and androst-4-ene-3,17-dione in human plasma has been described[66] in which the latter steroid, after separation, but prior to acetylation, was reduced to testosterone using sodium borohydride (eq 19). After acetylation and

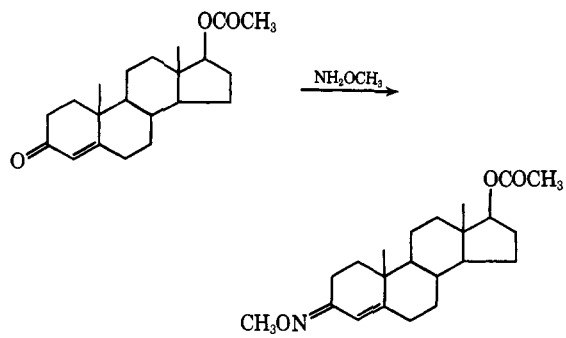


(19)

purification, inactive testosterone acetate was added and the mixture reduced to 3β-hydroxyandrost-4-ene 17β-acetate with sodium borohydride followed by more chromatographic purification. Tritiated steroids were used as indicators and [^{14}C]acetic anhydride as the acetylating agent. The final recovery for testosterone was given as 30–40% with a blank value of 8.7 ng in 10 ml of plasma, while corresponding figures for androst-4-ene-3,17-dione were a recovery of 10–25% and a blank value of 24.5 ng in 100 ml of plasma.

In a similar procedure for testosterone involving column, paper, and thin layer chromatography,[67] the 3β-hydroxyandrost-4-ene 17β-acetate reduction product was reoxidized back to testosterone acetate, achieving an overall recovery of 9%.

Another recent method[68] used enzymic reduction of androst-4-ene-3,17-dione to testosterone prior to acetylation and then formation of the *O*-methyloxime with *O*-methylhydroxylamine (eq 20).



(20)

(58) H. P. Shedl, P. S. Chen, Jr., G. Greene, and D. Redd, *J. Clin. Endocrinol. Metab.*, **19**, 1223 (1959).
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(64) M. A. Kirschner, M. B. Lipsett, and D. R. Collins, *J. Clin. Invest.*, **44**, 657 (1965).
(65) F. Dray, *Bull. Soc. Chim. Biol.*, **47**, 2145 (1965).
(66) M. A. Rivarola and C. J. Migeon, *Steroids*, **7**, 103 (1966).
(67) J. M. Saez, S. Saez, and C. J. Migeon, *ibid.*, **9**, 1 (1967).
(68) C. W. Bardin and M. B. Lipsett, *ibid.*, **9**, 71 (1967).

[^{14}C]Testosterone and [^{3}H]testosterone sulfate, and also [^{14}C]epitestosterone and [^{3}H]epitestosterone sulfate, were used as recovery indicators in a method for their analysis in the sulfate fraction of plasma of normal men.[69] Separation was achieved on a column of Celite. After separation the steroids were acetylated with [^{3}H]acetic anhydride and their sulfates were solvolyzed and then acetylated with [^{14}C]acetic anhydride. The acetates were purified by tlc on alumina, and then the quantity of each component was deduced from the ^{3}H/^{14}C ratios.

The use of isatin and 1,4-diaminoanthraquinone as dye markers in chromatography was described along with seven different chromatographic procedures. Blank values for testosterone and androsteronedione were given as 0.4 and 0.1 ng, respectively, with a coefficient of variation of 7–16%.

C. ALDOSTERONE

In a double isotope assay of aldosterone in urinary extracts with the combined use of thin layer and paper chromatography,[70] [1,2-^{3}H]aldosterone was added to the sample before isolation and subsequent acetylation with [1-^{14}C]acetic anhydride. The doubly labeled steroid acetate was purified by one-dimensional and then two-dimensional tlc, paper chromatography, and finally one-dimensional tlc to give an overall recovery of 20% with a blank value of 1 μg. In a later paper[71] isotope fractionation as a source of error in this method was discussed. In paper chromatography of [^{3}H]aldosterone [^{14}C]diacetate, isotope fractionation was shown to give rise to a considerable error in the ratio of ^{3}H to ^{14}C when the chromatographic peak was truncated asymetrically. Fractionation was even more pronounced in paper chromatography of mixtures of [^{3}H]- and [^{14}C]aldosterone, but it was not observed in mixtures of [^{14}C]aldosterone diacetate and [^{3}H]aldosterone diacetate.

Similar isotope derivative methods for aldosterone have been reported.[72–76] Modifications included gas chromatography[72] and formation of the benzylhydrazone after acetylation and purification.[73]

D. GESTOGENS

Double isotope derivative assays for progesterone and 20α-hydroxypregn-4-en-3-one have been reported.[77,78] Progesterone was enzymically reduced and measured as 20β-hydroxypregn-4-en-3-one using ^{14}C steroids as indicators and tritiated acetic anhydride as acetylating agent. Enhanced specificity of the method was obtained by forming the 3-*O*-methyloxime derivatives of the 20-acetoxypregn-4-en-3-ones[77] (see also ref 68); 50 to 100 ng of progesterone could be determined to within ±10% and 5 ng to within ±30%. In contrast other workers obtained a high blank value for the method due to a tritium impurity.[78]

E. THE STANOLS

A single isotope derivative dilution method was used for measuring 5α-cholestan-3β-ol in adrenal tissue.[79] After acetylation with [^{14}C]acetic anhydride and the addition of unlabeled acetate as diluent, the unsaturated sterols were converted into epoxy compounds by treatment with perbenzoic acid before final purification. The lower limit of detection was given as 7 μg.

F. ESTROGENS

Although extensive investigations into the determination of estrone and 17β-estradiol using ^{35}S- and ^{131}I-labeled pipsyl chloride have been reported,[43,44] several other workers have used [^{3}H]- or [^{14}C]acetic anhydride in a double isotope derivative method in conjunction with fluorescence measurements.[80] Traces of [6,7-^{3}H]estradiol were administered intravenously and the urinary metabolites obtained after hydrolysis with β-glucaronidase. These metabolites were separated by column chromatography and further purified by tlc prior to acetylation with [^{14}C]acetic anhydride. After the addition of inactive carrier acetates and further purification by tlc, a second quantity of carrier was added, and the compounds were recrystallized until a constant isotopic ratio was attained. The procedure was checked by adding inactive estrone and estriol to the sample extract and repeating the purification and isolation. Prior to acetylation the estrone fraction was reduced to estradiol, purified, and analyzed as its ^{14}C diacetate. Radio-gas chromatography was used for detecting extraneous radioactivity but none could be found. Attempts to use fluorimetry for the determination of estrone and estriol before the addition of carrier, however, were unsuccessful in this case. Using similar techniques Barlow[81] successfully measured the fluorescence with sulfuric acid, specificity being achieved by adding tritiated estrone, 17β-estradiol, and estriol to the urine hydrolysate prior to purification and acetylation. The lower limit of sensitivity was given as approximately 0.2 μg per 24 hr of excretion.

Finally, in this section on acetyl derivatives, 4-hydroxy-3-methoxymandelic acid in serum and urine was separated by electrophoresis and then acetylated with [1-^{14}C]acetic anhydride using trifluoroacetic acid catalyst.[82] The acetate was repurified by electrophoresis and then compared with a standard which had been treated in the same manner. Assay was by liquid scintillation counting. Later it was shown that sensitivity could be greatly improved by the use of [1-^{3}H]acetic anhydride in acetonitrile.[83]

Several other labeled reagents have been used to measure hydroxylic compounds. [^{3}H]Dimethyl sulfate gave a qualitative identification of estradiol and estrone in avian plasma[84] (eq 21).

(69) F. Dray, I. Mowszowicz, and M. J. Ledru, *Steroids*, **10**, 501 (1967).
(70) T. J. Benraad and P. W. C. Kloppenborg, *Clin. Chim. Acta*, **12**, 565 (1965).
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(72) B. Kliman, "Gas Chromatography of Steroids," M. B. Lipsett, Ed., Plenum Press, New York, N. Y., 1965, p 101.
(73) R. E. Peterson, "Aldosterone," E. E. Banlion and P. Robel, Ed., Blackwell, Oxford, 1964.
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(81) J. J. Barlow, *Anal. Biochem.*, **6**, 435 (1963).
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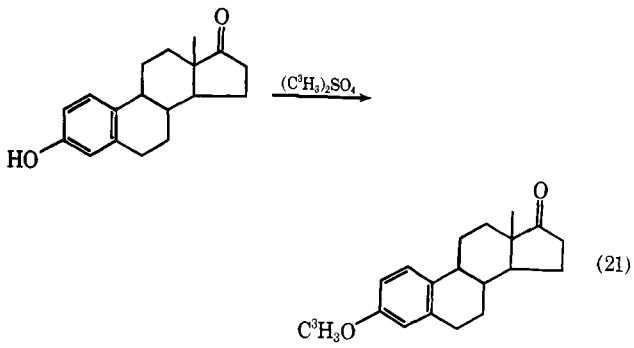
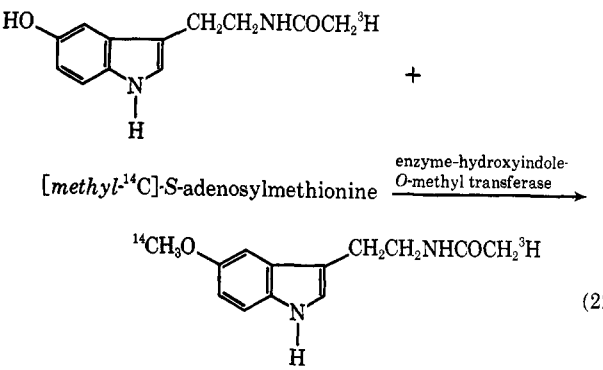
(83) L. P. O'Gorman, *ibid.*, **23**, 247 (1969).
(84) J. E. O'Grady and P. J. Heald, *Nature*, **205**, 390 (1965).



(21)

A double isotope derivative technique was used, and after methylation and destruction of excess [^{3}H]-dimethyl sulfate, authentic ^{14}C-methylated estradiol and estrone were added to the mixture which was subsequently separated and purified by repeated thin layer and column chromatography until a constant isotopic ratio was obtained. Although the method as described was qualitative only, there appear to be no objections to its use for the quantitative determination of submicrogram quantities of materials of this type, provided that the reagents were standardized.

A double isotope method for the estimation of tissue levels of *S*-adenosylmethionine used the ^{14}C-labeled compound and [*acetyl*-^{3}H]-*N*-acetylserotonin as labeled reagent.[85] An enzymic reaction was utilized in which the hydroxyl group of [acetyl-^{3}H]-*N*-acetylserotonin was methylated by transfer of the ^{14}C-methyl group from *S*-adenosylmethionine, to give [*methoxy*-^{14}C-*acetyl*-^{3}H]melatonin (eq 22). A simple

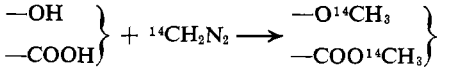


(22)

isotope dilution procedure was used involving addition of a known amount of [*methyl*-^{14}C]-*S*-adenosylmethionine to the tissue extract prior to enzymic reaction.

Determination of hydroxyl compounds using [^{36}Cl]-3-chloro-4-methoxybenzoyl chloride was described.[86] This method is reviewed later in section VII, dealing with measurement of amino groups, and so will not be discussed further here.

The superficial hydroxyl and carboxyl groups of carbon blacks were measured using ^{14}C-labeled diazomethane.[87]



(23)

The sample of black was dispersed in a toluene solution of $^{14}CH_2N_2$ and, after evolution of nitrogen, was dried, Soxhlet extracted, and then redried before chemical and radiochemical analysis. This procedure gave the total hydroxyl and carboxyl contents of the black surface. The methyl esters in one portion of the ^{14}C-methylated product were then hydrolyzed, and residual methoxy groups were determined as before by combustion, followed by absorption of the $^{14}CO_2$ in ethanolamine–methoxyethanol prior to measurement of activity by liquid scintillation counting. The carboxyl content was obtained from the difference between the two determinations.

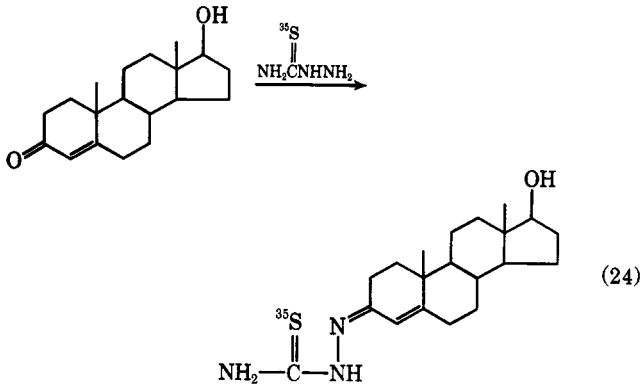
A general method of analysis for sterols, amines, acids, and aldehydes using various ^{131}I-labeled reagents has been described.[88] Chromatographic separation and location of various selected pairs of structurally related, labeled derivatives were examined. [^{131}I]-*p*-Iodobenzoyl chloride was used for the simultaneous determination of cholestanol and cholesterol and *o*- and *m*-toluidine. For acetic and propionic acids, [^{131}I]-*p*-iodoaniline was used, while [^{131}I]-*p*-iodophenylhydrazine was the reagent of choice for *o*- and *m*-nitrobenzaldehyde. An automatic scanning and recording apparatus was developed for measuring the distribution of the associated γ activity in the chromatography column. After such a separation the column was allowed to run dry and counts were made at 1-cm intervals down its whole length. It was then sectioned so that each component was divided into six equal portions and reassayed. By this method it was shown that cholesterol was contaminated with less than 1.4% of cholestanol. A scanning technique of this type was obviously only applicable to γ- or strong β-emitting isotopes because of absorption by the glass walls of the column, and the use of this particular procedure appears to have been very restricted since the advent of radio glc and tlc chromatogram scanning using windowless or very thin window GM tubes for the more recent low-energy, longer half-life β emitters.

IV. Analysis of Carbonyl Compounds

Several workers have used [^{35}S]thiosemicarbazide as a reagent for steroids containing reactive carbonyl groups, because of the satisfactory chromatographic properties of the derivatives. Unlike the pipsyl or acetic anhydride reagents, thiosemicarbazide is not affected by water and reacts quantitatively with keto steroids in crude extracts from plasma, whereas with the former reagents, greater purification is required prior to derivative formation. The thiosemicarbazides tend to absorb strongly on paper and silica gel, however, necessitating high carrier levels and very careful elution, and because of this, high blank values are sometimes obtained with this reagent. This may be due in part to inadequate separation of excess reagent before addition of the carrier.

The development of this compound as a labeled reagent has been fully described.[89] It was originally used for the estimation of testosterone in peripheral blood,[90] by a double isotope procedure employing [1,2-^{3}H]testosterone as indicator and [^{35}S]thiosemicarbazide as reagent. A sample of plasma containing added indicator was extracted and dried before formation of the [^{35}S]thiosemicarbazone (eq 24). The derivative was purified by thin layer and paper chromatography; then hydrolysis with pyruvic acid was used to assist in removal of impurities and to selectively hydrolyze any thiosemicarbazone formed at position 20. The dried eluate was then acety-

(85) R. J. Baldessarini and I. J. Kopin, *Anal. Biochem.*, 6, 289 (1963).
(86) P. Sorensen, *Anal. Chem.*, 27, 388 (1955).
(87) E. Papirer and J. B. Donnet, *Bull. Soc. Chim. Fr.*, 6, 2033 (1966).
(88) W. M. Stokes, F. C. Hickey, and W. A. Fish, *Anal. Chem.*, 27, 1895 (1955).
(89) J. F. Tait, B. Little, S. A. S. Tait, A. Riondel, C. Flood, E. Joachim, and M. Gut, *Advan. Tracer Methodol.*, 2, 227 (1963).
(90) A. Riondel, J. F. Tait, M. Gut, S. A. S. Tait, E. Joachim, and B. Little, *J. Clin. Endocrinol. Metab.*, 23, 620 (1963).



(24)

ated and the 2,4-diacetylthiosemicarbazone of testosterone purified by further thin layer and paper chromatography. The amount of testosterone in plasma was calculated from the ratio of the ^{35}S to ^{3}H activity of the acetyl derivative. A known amount of testosterone was taken through the method and the [^{35}S]-2,4-diacetylthiosemicarbazone of [1,2-^{3}H]testosterone used as a permanent standard. A mean overall recovery of 7% was obtained with a coefficient of variation of 5.5%. The method was also used to determine epiandrosterone, androsterone, etiocholanolone, and epietiocholanolone.

The same workers[91] later applied an identical procedure to the estimation of progesterone in human peripheral blood. In this case the 3,20-bis(thiosemicarbazone) of progesterone was formed and hydrolyzed to progesterone 3-thiosemicarbazone with pyruvic acid. A mean overall recovery of 3% was obtained, with a coefficient of variation of 7.5% for replicate estimations. The blank value was reported as 2 ng.

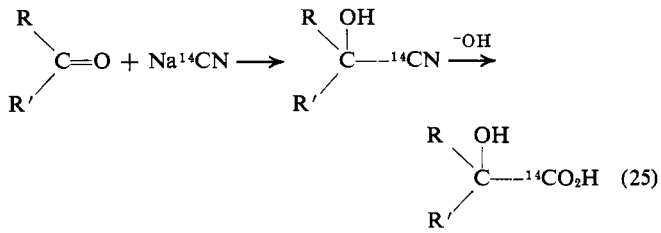
A similar procedure was used for androstenedione, with an overall recovery of 4.5% after the fourth chromatogram and 1.2% after the fifth.[92,93] The coefficient of variation was given as 4.3%.

A modification of this procedure[94] for estimation of plasma testosterone was important since recovery was increased by a factor of 3, to 22%. The testosterone was partially purified by thin layer chromatography and then acetylated with acetic anhydride in pyridine. This testosterone 2,4-diacetate was treated with [^{35}S]thiosemicarbazide by the usual procedure and the [^{35}S]thiosemicarbazone purified by chromatography without pyruvic acid hydrolysis. Further acetylation was carried out after the second chromatography of the labeled derivative. These semicarbazone acetates had different chromatographic characteristics facilitating further purification. For example, testosterone 3-thiosemicarbazone 2,4-diacetate could be separated from testosterone 3-monothiosemicarbazone.

Prior to the development of [^{35}S]thiosemicarbazide there was no general method available for the estimation of steroids not containing acetylatable or methylatable hydroxyl groups. However, about the same time sodium borotritide was used for the reduction of progesterone followed by partial reoxidation using manganese dioxide.[95,96] Because of the instability of this reagent, the reduction was carried out at low temperatures (−15°). The steroid was partially purified before reduction, necessitating the addition of [4-^{14}C]progesterone indicator to the sample at the beginning of the analysis. The tritiated reagent was standardized with pure progesterone and [4-^{14}C]progesterone. A recovery of 22.5 ± 4.5% was obtained at the 0.1-μg level, which was increased to 48.9 ± 14.3% at the 1.0-μg level, with a standard deviation of only 2%.

Sodium borotritide had the advantage of reducing only aldehydes and ketones and therefore had a degree of specificity comparable to [^{35}S]thiosemicarbazide. Partial reoxidation of the tritiated material removed labile tritium, thus enabling the derivatives to be extracted and purified more easily in addition to simplifying location and counting.

Labeled reagents have also been used for the determination of sugars and polysaccharides. In 1951 Isbell[97] described the determination of reducing end groups in polysaccharides using sodium [^{14}C]cyanide (eq 25) and suggested this as a simple means for estimation of their molecular weight. Later



(25)

the method was developed for structural analysis of clinical dextrans, using periodate oxidation with isotope dilution techniques.[98] These dextrans were bacterial polysaccharides consisting of anhydro-D-glucose units connected for the most part by 1,6′ linkages, with occasional branching at carbon atoms 3 and 4. The polymeric aldehydes resulting from periodate oxidation of the dextrans were hydrolyzed and D-glucose and D-erythrose from the 1,3′ and 1,4′ linkages were estimated as follows. The nitriles produced by reaction of hydrolysis products with sodium [^{14}C]cyanide in the presence of barium carbonate were converted *in situ* to aldonic acids, which were isolated as their salts after addition of sodium D-glycero-D-guloheptonate and potassium D-arabonate as carriers. These materials were counted and then compared with the values obtained for standard solutions of the two sugars. The percentages of 1,6′ linkages were determined on separate samples from the amount of formic acid produced by oxidation with sodium metaperiodate. This work extended the usefulness of periodate oxidation techniques for the structural analysis of dextrans, making it more sensitive, but the method suffered from the disadvantage (relatively speaking) of being a lengthy procedure and requiring special equipment.

Prior to this a procedure was devised for standardization of sodium [^{14}C]cyanide using the reaction with a reducing sugar,[99] and from this they developed a general method for determination of small quantities of reducing sugars and for the study of polysaccharides.[100] The sugar or polysaccharide was allowed to react in a buffered solution with sodium

(91) A. Riondel, J. F. Tait, S. A. S. Tait, M. Gut, and B. Little, *J. Clin. Endrocrinol. Metab.*, **25**, 229 (1965).
(92) R. Horton, *ibid.*, **25**, 1237 (1965).
(93) R. Horton and J. F. Tait, *Proc. Int. Congr. Endocrinol., 2nd., 1964*, 268 (1965).
(94) N. Y. Lim and R. V. Brooks, *Steroids*, **6**, 561 (1965).
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(96) C. A. Woolever, *Proc. Int. Congr. Endocrinol., 2nd, 1964*, 287 (1965).
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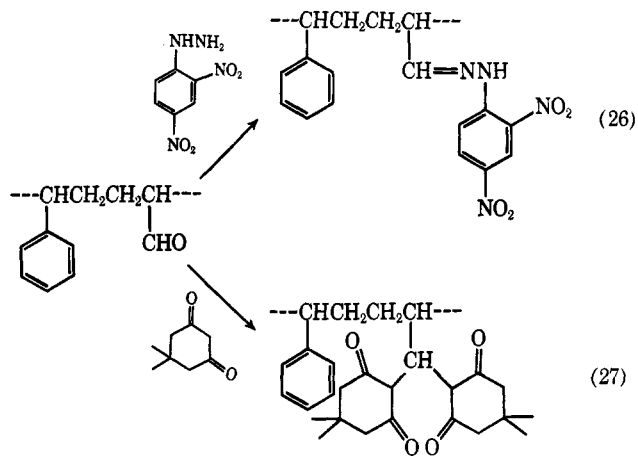
(98) J. D. Moyer and H. S. Isbell, *Anal. Chem.*, **29**, 1862 (1957).
(99) J. D. Moyer and H. S. Isbell, *ibid.*, **29**, 393 (1957).
(100) J. D. Moyer and H. S. Isbell, *ibid.*, **30**, 1975 (1958).

[^{14}C]cyanide, previously standardized by reaction with D-glucose. The excess cyanide was volatilized as H^{14}CN and the ^{14}C in the residue determined by measurement of the radioactivity. All the aldose monosaccharides and a few disaccharides gave results in accord with the reaction of 1 mol of sugar with 1 mol of cyanide. Under the conditions used, some sugars and polysaccharides combined with more than 1 equiv of cyanide. In the analysis of these materials it was necessary to have a control with a known quantity of the material in question. The high results with some materials were ascribed to cleavage and hydrolysis reactions arising from enolization. They reported a standard deviation of ±1.4% for all their determinations, and a satisfactory reproducibility for samples from 0.04 to 0.2 mg of glucose.

The same reagent was used for determination of glycogen, but nonspecific reactions led to abandonment of this procedure.[101]

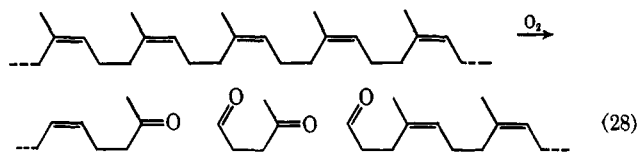
Since high specific activity sodium [^{14}C]cyanide and tritiated sugars are now available, there appear to be no objections to the same reagent being used in a double derivative analysis for estimation of nanogram quantities of reducing sugars.

Radiotracer techniques have been successfully applied to the quantitative estimation of ketone and aldehyde groups formed during the oxidative scission of natural rubber.[102,103] Under suitable conditions 2,4-dinitrophenylhydrazine and dimedone were shown to react quantitatively with the aldehyde groups of a styrene–acrolein copolymer of known composition (eq 26 and 27). The nitrogen content and uv spectrum



of the polystyrene–acrolein 2,4-dinitrophenylhydrazone were measured and compared with a value determined radiochemically using [^{14}C]-2,4-dinitrophenylhydrazine. The shape of the spectrum indicated a residual background absorption due to light scattering by the slight turbidity of the solutions. The absorption due to the phenylhydrazone at 350 mμ was found from the difference between the experimental curve and the interpolated scatter curve. Because of the uncertainty of the exact position of the background absorption curve, the values obtained by this spectroscopic method were only accurate to ±10%.

The technique was first used for measurement of rubber bound aldehyde groups in natural rubber latex, using [^{14}C]-dimedone.[102] In particular, the clonal variation of aldehyde content of the latex was studied in relation to the storage-hardening behavior of the different samples. After treatment of the latex with labeled reagent, in which two molecules of dimedone condensed with one aldehyde group, unreacted reagent was removed by repeated precipitation of the rubber from solution. The radioactivity was measured either by Schöniger combustion to $^{14}CO_2$ and absorption in ethanolamine–methoxyethanol or direct swelling in phosphor solution prior to liquid scintillation counting. The technique was later applied to measurement of the oxidation scission products of natural rubber, with a view to elucidating the scission mechanism.[104] Various mechanisms had been proposed,[105–108] each of which predicted the formation of rubber bound ketone and aldehyde groups and lower molecular weight carbonyl compounds during each scission event (*e.g.*, eq 28). After oxidation to a known extent under carefully



controlled conditions, rubber-bound ketone and aldehyde groups were measured by direct labeling with [^{3}H]-2,4-dinitrophenylhydrazone and [^{14}C]dimedone to give total carbonyl and aldehyde contents, respectively; ketone groups were obtained by difference. Unreacted reagents were removed by precipitation or, in the case of a vulcanized rubber, by Soxhlet extraction. The rubber 2,4-dinitrophenylhydrazones were combusted to tritiated water and measured by liquid scintillation counting. Dimedone derivatives were swollen directly in phosphor solution. An in-vial microcombustion technique was also used for submilligram quantities of labeled rubbers, microtomed as sections from various aged samples.[108]

Low molecular weight scission products predicted by the various mechanisms, *e.g.*, acetonylacetone,[105] levulinaldehyde, formaldehyde, levulinic acid, etc.,[106,107] were all estimated by an isotope derivative dilution technique involving formation of the [^{3}H]-2,4-dinitrophenylhydrazone and addition of a large, known excess of unlabeled carrier. In the case of levulinic acid analysis,[109] a second benzaldehyde derivative was formed from unreacted 2,4-DNPH, and the two were separated by selective extraction in a basic solution. After repeated recrystallization to constant specific activity, the samples were combusted to tritiated water before counting because of severe quenching of fluor solutions by the highly colored derivatives. The method was used regularly for determination of microgram quantities of scission products but could equally be applied to much lower levels, in the nanogram range, by use of higher specific activity reagents.

Since the concomitant formation of ketone groups with scission events was shown to be quantitative in a soluble rubber, by comparison with viscometric and osmometric measurements, the method was used in an extensive investigation of the effects on the scission efficiency of oxygen, ϵ, of antioxidants and various model sulfides structurally related

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(108) F. R. Mayo, *Ind. Eng. Chem.*, **52**, 614 (1960).
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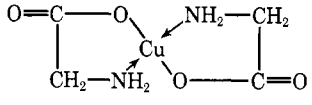
to the mono-, di-, and trisulfidic cross-links present in sulfur vulcanizates of natural rubber. The parameter ϵ is defined as the number of molecules of oxygen required to produce one main chain scission event. In simple peroxide and sulfur vulcanized rubbers, the results were compared with those obtained from stress–strain measurements, in which changes in modulus and hence network density were related to the number of scission events.[110] The radiochemical method had the advantage that it could be applied to silica and black filled vulcanizates with equal success, whereas use of the stress–strain method was precluded because of filler effects. In addition, in sulfur vulcanizates where oxidative cross-linking was known to occur, modulus measurements gave the net result of main chain and cross-link scission and cross-link formation, whereas the tracer method measured main chain scission only. For obvious reasons, viscometric and osmometric measurements could not be applied to vulcanizates or gel systems. Technological applications of the method included a study of the variation of oxidative degradation with depth in various tires and aged samples.

A similar procedure has been described for the determination of carbonyl groups in pretreated polyethylene film.[111] Carbonyl groups were introduced into the film by exposure to X-rays in an oxidizing atmosphere or by immersion of the film in chromic acid solution. The films were washed and immersed in a fresh solution of [^{14}C]-2,4-dinitrophenylhydrazine until hydrazone formation was complete. Two methods were used to determine the hydrazone, (i) direct measurement of radioactivity and (ii) extraction of the hydrazine with pyridine, and titration with tetrabutylammonium hydroxide in methanol–2-propanol medium. The volumetric method gave reproducible results only with large samples (>100 cm^2). The variation of carbonyl group content was determined with time and temperature of treatment and with different radiation doses.

V. Analysis of Carboxyl Compounds

Several isotopic methods of carboxyl group determination have utilized the formation of salts with heavy metals such as copper, cobalt, cerium, and silver. In particular, amino acids form chelate compounds as, for example, the copper salt of glycine which is formed by heating copper oxide with an aqueous solution of glycine.



Blackburn and Robson[112,113] utilized this property in their radiochemical method for micro estimation of α-amino acids separated on paper partition chromatograms. Sections cut from the chromatograms were immersed overnight in phosphate solution, and then a suspension containing [^{64}Cu]-copper phosphate ($^{64}Cu_3(PO_4)_2$) was added. After dissolution and filtration, aliquots of the copper–amino acid complex were evaporated to dryness for counting. The limits of detection by this procedure were given as 1 μg of α-amino nitrogen with an accuracy of $\pm 3\%$ or 2 μg with a corresponding figure of $\pm 2\%$. The amino content of salmine sulfate has been determined completely by this method.[113] A major drawback, however, would appear to be the very short half-life of ^{64}Cu (12.8 hr). An almost identical procedure has been described using $^{64}Cu(OAc)_2$ instead of $^{64}Cu_3(PO_4)_2$ for complex formation.[114]

Several workers[115–118] have described the radiochemical determination of carboxyl groups in cellulose and oxycellulose. A small quantity of these groups is formed during bleaching of cellulose, and, since they have a profound effect on such properties as the reactivity of the fiber toward basic dyes, in addition to the ultimate strength of the material, their detection in even low concentrations is of utmost importance. One method used cerous acetate ($^{144}Ce(OAc)_3$) as the labeling agent.[115] The cellulose was first treated with dilute acid to remove other mineral elements already present in the fiber; then after a thorough washing, it was shaken for about 14 hr with a solution of [^{144}Ce]cerous acetate. A long reaction time was necessary to ensure complete penetration of the fiber by reagent. After another washing to remove unreacted [^{144}Ce]cerous acetate, it was dried and the activity measured using a Geiger–Müller tube. The second washing was carried out relatively quickly in order to minimize the effects of slow hydrolysis of the ^{144}Ce. By comparison with cellulose samples of known composition they were able to establish a relationship between measured radioactivity and the number of acid groups present.

Cobalt-60 and silver-110 salts were used for the determination of carboxyl and carbonyl groups in cellulose oxidized with either periodate (oxycellulose) or periodic acid (acid oxycellulose).[116,117] A desized, unbleached rayon fabric was oxidized with sodium or potassium periodate and then treated with sodium perchlorate to convert the aldehyde groups to carboxyls. After washing and drying, samples of cellulose were immersed in [^{60}Co]cobaltous nitrate ($^{60}Co(NO_3)_2$) or [^{110}Ag]argentous nitrate ($^{110}AgNO_3$) solution (1.5 μCi/ml). Unreacted reagent was then removed by washing and the sample dried and assayed. Good agreement was obtained between carboxyl groups calculated from the adsorbed ^{60}Co and the consumption of periodate.

In the case of acid oxycelluloses (using periodic and perchloric acids in place of their salts) having two carboxyl groups in the 2,3 position, bivalent ^{60}Co atom was bound to two carboxyls. Experiments were also carried out with uranyl salts, the results of which agreed with the ^{60}Co results in the case of uranyl nitrate, $UO_2(NO_3)_2 \cdot 6H_2O$, but not with the acetate, $UO_2(OAc)_2$, probably owing to hydrolysis of the latter.

The same principles were used for determination of carboxyl groups in cellulose by measurement of the decrease in radioactivity of a solution containing calcium-45 acetate.[118] In the case of unbleached pulp, both the carboxyl groups of cellulose and the sulfonic acid groups of lignin reacted with the ^{45}Ca ions, but this proved advantageous since it meant that the radiometric method could be applied to follow the

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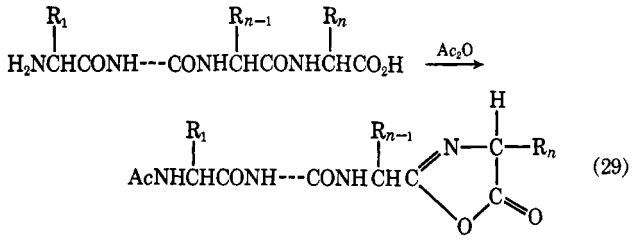
change in adsorption during hydrosulfite bleaching, the maximum of the adsorption curve corresponding to the moment when lignin began to pass into solution.

A novel method for determination of C-terminal amino acids in polypeptides by selective tritium labeling has been described.[119a] The selective deuteration or tritiation was based upon the C-terminal oxazolone formation in peptides by the action of acetic anhydride or dicyclohexylcarbodiimide (eq 29). The oxazolone was subjected to base-catalyzed,



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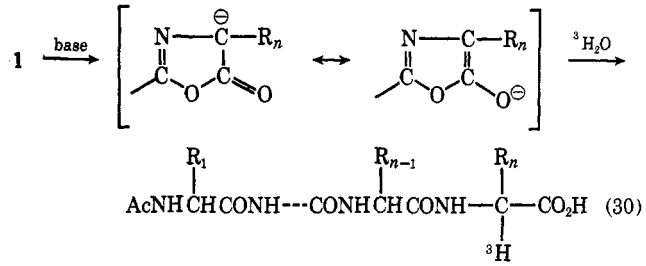
hydrolytic ring-opening in ^2H_2O or ^3H_2O to give a peptide having a labeled C-terminal amino acid (eq 30).



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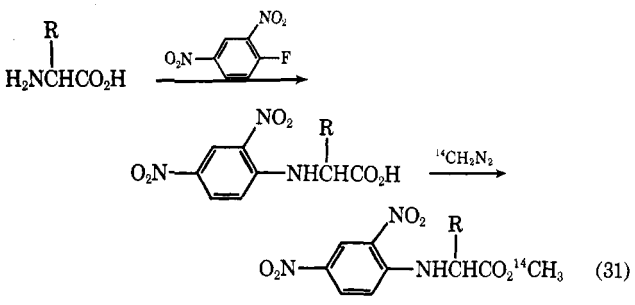
Finally, the peptide was hydrolyzed into its constituent amino acids which were separated by normal chromatographic procedures. This method has been successfully used to determine the C-terminal amino acids of angiotensin and beef insulin. Careful purification of the labeled peptide was necessary prior to the final hydrolysis because of the relatively large number of labile hydrogen atoms present in addition to the nonlabile position selectively labeled. A critical evaluation of the method has recently been reported.[119b]

An ultramicro determination of amino acids as the [^{14}C]-methyl esters of their 2,4-dinitrophenyl derivatives has been described.[120] The esters were prepared by treating the amino acids with unlabeled 1-fluoro-2,4-dinitrobenzene, followed by methylation using [^{14}C]diazomethane (eq 31). The de-



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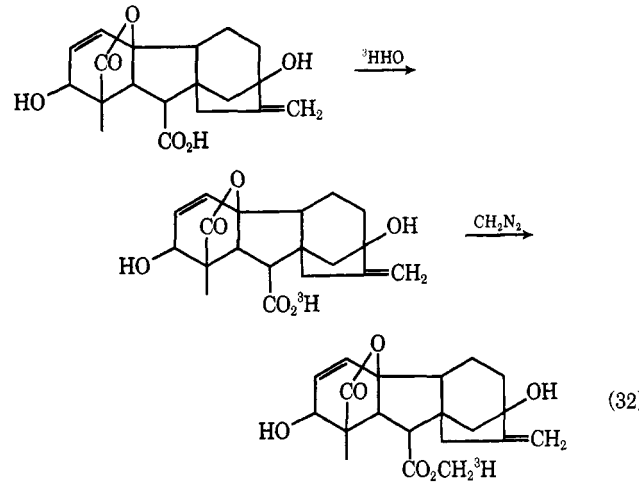
rivatives were separated by one-dimensional chromatography on formamide impregnated paper, and, according to the amino acids to be separated, three sets of conditions were described for time of impregnation of the paper and the solvent system used. The method was tested on a prepared mixture of 21 amino acids and on an insulin hydrolysate; the limit of detection was given as 10^{-4} μmol per component.

Several other radiochemical methods of carboxyl group determination have utilized [^{14}C]diazomethane. Thus gibberellins in fermentation liquors were measured by derivative labeling with [^{14}C]diazomethane and by isotopic dilution analysis with tritium-labeled gibberellins.[121,122] In the earlier work,[121] the [^{14}C]methyl esters of gibberellic acid and gibberellin A were separated by paper chromatography and measured with a radiochromatogram scanner. If necessary, the sensitivity and accuracy of the method could be increased by extraction of the chromatogram spots, followed by liquid scintillation counting. Alternatively, gibberellic acid was labeled on the hydrogen of the carboxyl group by exchange with tritiated water. Treatment with diazomethane then gave a tritium labeled ester (eq 32).



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In pilot plant studies of the separation of gibberellins from process liquors, step-by-step recovery was followed by adding tritium-labeled (Wilzbach procedure) gibberellic acid and assaying successive samples by liquid scintillation counting, a single sample being isolated to determine the activity of the final product.

In later studies a double isotope derivative technique was used in which tritium-labeled gibberellins were treated with [^{14}C]diazomethane of known specific activity and the gibberellin esters purified by paper chromatography until a constant ratio of ^{3}H to ^{14}C activity was obtained.

In a general micro method for the determination of acidic substances of biological significance, treatment with [^{14}C]-diazomethane under mild conditions resulted in complete methylation of nonesterified fatty acids in plasma and of bile acids in bile.[123] The [^{14}C]methyl esters of specific bile acids were shown to be isolable by thin layer chromatography free from other active substances. A similar procedure was also described for determination of free acids in mixtures

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(123) H. S. Sodhi and P. D. S. Wood, *Proc. Soc. Exptl. Biol. Med.*, 113, 714 (1963).

of lipids.[124,125] In addition, hydroxyl and amino groups were esterified with [^{3}H]- or [^{14}C]acetic anhydride, and the mixture was separated into groups by column chromatography. Fractions were further separated on paper and analyzed by radiochemical and spectrophotometric techniques. Specific details were given for the chromatographic separation of mixtures of lipids and for the separation of mixtures of long-chain alcohol acetates, amine acetates, acetyl diglycerides, diacetyl monoglycerides, and acetoxyethylamides.

Use of [^{14}C]diazomethane for measurement of superficial hydroxyl and carboxyl groups of carbon blacks[87] has already been described in an earlier section.

As an alternative to the complicated and expensive procedure of direct esterification using labeled diazomethane, a process known as "tritiating methylation" may be used.[126,127] It has been applied to the quantitative chromatographic analysis of organic acids.[128] The anhydrous acid was exchanged with tritiated water under nonaqueous conditions and esterified with unlabeled diazomethane in the normal way (eq 32a), and the esters were separated by thin layer chromatography.

$$RCOO^3H + CH_2N_2 \longrightarrow RCOOCH_2{}^3H + N_2 \quad (32a)$$

The spots were then located and scraped from the plate for measurement of activity. Recoveries of 95% were reported in the 1–5-mg range and results were still satisfactory in the microgram range if carrier esters were added before chromatography. The total yield of incorporation of tritium per sample was found to be small and variable (1–7% of the total activity of the tritiated water), but this was not due to incomplete reaction since no significant difference in the incorporated tritium activity was found when the time of tritium exchange with stearic acid was varied by a factor of 15. Although there was no reference to the fact in the original paper, it would appear that a kinetic isotope effect was operating during the exchange reaction. When absolute information was required, a known amount of an internal standard was added before tritiation, resulting in a reproducibility within 5%. From various test mixtures it was shown that stronger acids such as tartaric, benzoic, and phthalic acids could be studied directly with a weak acid such as stearic acid. There were also indications that phenolic compounds could be treated in the same way, although this was possibly due to direct acid-catalyzed exchange of tritium between the tritiated water and diazomethane, in addition to exchange of the acids with tritiated water.[129]

A specific, sensitive method for determining carboxyl end groups in Nylon 66 used esterification by [^{14}C]methanol with boron trifluoride as catalyst.[130] After esterification (eq 33) the polymer was precipitated, washed, dried, and assayed by liquid scintillation counting of $^{14}CO_2$ from an oxygen flask combustion. It was necessary to subtract a degradation blank from the carboxyl end-group value, the apparent carboxyl content increasing by 300% over a reaction time of 2 hr. Normally, however, 10 min of labeling time was adequate, resulting in a degradation blank of 4.5 μequiv of carboxyl end groups per gram of polymer. The method was compared with the procedure in which samples were titrated with potassium hydroxide in hot benzyl alcohol to a phenolphthalein end point,[131] and gave slightly higher results for most samples. A standard deviation of approximately ±1.5 μequiv/g was obtained for 20–50-mg samples. Another esterification technique was developed for micro determination of total fatty acids in biological samples.[132] Evaporated chromatography eluates containing 0.03–50 μmol of acids were dissolved in chloroform and shaken with alkaline [*methyl*-^{3}H]methanol for 1 hr at room temperature. Then the mixture was neutralized, washed with water, and evaporated to dryness. The residue was dissolved in fluor solution and counted in a liquid scintillation counter. The results agreed well with those obtained by glc.

$$H_2N(CH_2)_6NH\text{---}CO(CH_2)_4CO_2H \xrightarrow{^{14}CH_3OH} H_2N(CH_2)_6NH\text{---}CO(CH_2)_4CO_2{}^{14}CH_3 \quad (33)$$

A technique not strictly involving formation of a labeled derivative has been described for the determination of cholinesterase activity using sodium [^{14}C]bicarbonate.[133] The method was based on the principle used in manometric techniques, the bicarbonate reacting with the acetic acid liberated by enzymic hydrolysis of acetylcholine. The resulting $^{14}CO_2$ was collected in an ethanolamine–2-methoxyethanol absorbant and measured by liquid scintillation counting. The method was also applicable to determination of cholinesterase inhibition and was much more sensitive than the corresponding nonisotopic procedure involving measurement of the volume of gas evolved.

The use of ^{36}Cl derivatives to determine acetylatable compounds by isotope dilution[86] was found to offer so many advantages that the method was extended to systems characterized by other functional groups.[134] The principle was applied to determination of carboxylic acids, acid chlorides, and anhydrides. The compound to be analyzed was quantitatively converted to the *p*-chloroanilide (eq 34) which was then determined by an ordinary isotope dilution analysis using the appropriate ^{36}Cl-tagged *p*-chloroanilide. Quantitative

$$RCO_2H + p\text{-}H_2NC_6H_4Cl \longrightarrow p\text{-}RCONHC_6H_4Cl + H_2O \quad (34)$$

conversion of acid chlorides and anhydrides to anilides was straightforward. Carboxylic acids were usually first converted to the acid chloride, and, provided this latter compound was nonvolatile, the two-step reaction could be quantitative. Alternatively, when *p*-chlorophenylphosphazo-*p*-chloroanilide ($ClC_6H_4N{=}PNHC_6H_4Cl$) was used under the proper reaction conditions, carboxylic acids could be quantitatively converted in one step to *p*-chloroanilides which were easily purified and had sharp melting points.[135–138] For the analysis of acetic, benzoic, and stearic acids and acetic anhydride, all of known purity, a statistical error corresponding to a standard deviation of 0.6–0.7% was claimed. For stearic acid,

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which formed a nonvolatile acid chloride, both the one- and two-step methods of *p*-chloroanilide formation were used, the latter procedure giving a slightly higher recovery. For acids generally, a mixture of *p*-chloroaniline and the phosphazo compound was found to be best. A disadvantage of the method was that it was not applicable to dicarboxylic acids because of the formation of half-anilides or -imides. Experiments with succinic acid, for example, gave conversions of approximately 60%.

p-Bromo-α-haloacetophenones have been suggested as reagents for the estimation of fatty acids by isotope derivative dilution.[139] The radioactive reagent, labeled with ^{14}C in the carbonyl group, was used to determine the yield of phenacyl myristate, and an average yield of 60% was obtained. It was optimistically suggested that once yields were established for decanoic, lauric, palmitic, stearic, oleic, linoleic, and linolanic acids, a correction factor could be applied to determine these and other fatty acids by isotope derivative analysis.

VI. Analysis of Thiol Compounds

The measurement of thiol groups, particularly in substances of biological origin, is of prime importance since it has been shown that the presence of these substituents is often necessary for the retention of biological activity.

It has been shown that for some enzymes, a certain fraction of the substituent thiol groups must be maintained as such, in the "reduced" form, in order that the catalytic activity remain unimpaired. Activity has been correlated with thiol content for a number of enzymes.[140] Physiologically, these groups appear to play an important role in, for example, the secretion of urine, possibly in iron metabolism, and in the control of circulatory dynamics in shock.[141]

Traditional methods of thiol group determination utilize its broad spectrum of chemical reactivity such as oxidation to disulfide, mercaptide formation, or alkylation.[142] In particular, mercaptide formation has been used in conjunction with amperometric titration[143] and spectrophotometric procedures.[144] All these traditional methods are limited, however, in that milligram quantities of protein are normally required, and, in the case of spectrophotometric techniques, other substances which absorb at or near 250 mμ, *e.g.*, adenine nucleotides, may interfere with the method.

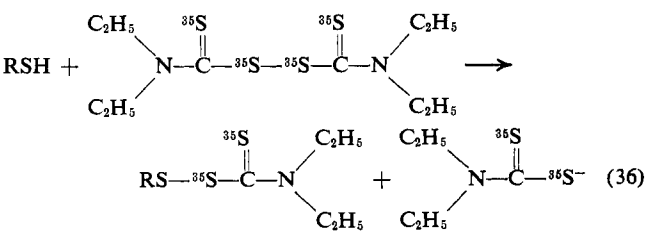
These disadvantages were easily circumvented by the use of radiolabeled reagents, the sensitivity of the method being theoretically limited only by the specific activity of the reagent used. For the determination of total thiol content of proteins, preliminary denaturation of the protein was usually essential[145] to produce maximum chain separation and hence accessibility to the thiol groups by reagent.

A sensitive gel filtration method is available for the determination of protein thiol groups with carboxyl-labeled [^{14}C]chloromercuribenzoic acid ([^{14}C]CMB).[146] For oxy- and (carbon monoxy)hemoglobin, monoamine oxidase, and yeast cytochrome *c*, the [^{14}C]CMB was shown to react quantitatively with protein thiol groups (eq 35, R = protein, peptide chain, etc.) in less than 1.5 min at 30°. Unreacted reagent was separated from labeled protein by gel filtration using a Sephadex-G25 column. After protein determination, aliquots were counted in a low-background gas-flow counter. Samples of [^{14}C]CMB were counted simultaneously under a similar set of conditions. From the published data the lowest level of protein actually assayed appeared to be 30–40 μg in 0.20 ml of incubation mixture. In all cases, results in close agreement with known literature values were obtained. Horse heart cytochrome *c* and lysozome, which have no thiol groups, did not bind the mercurial reagent.

$$RSH + p\text{-}HO^{14}COC_6H_4HgCl \longrightarrow p\text{-}HO^{14}COC_6H_4HgSR + HCl \quad (35)$$

Mercaptide formation has been used in a direct labeling technique for estimation of thiol and disulfide groups in insoluble proteins, such as wool fibers, using either [^{203}Hg]-phenylmercuric acetate or [^{14}C]methylmercuric iodide.[147] Either the decrease in radioactivity of the reagent solution (samples > 1 mg) was measured or the uptake of radioactivity by the protein assayed either directly or after combustion. The latter method was used for single wool fibers weighing 50–1000 μg, direct γ-ray counting of the ^{203}Hg isotope then being employed. Samples containing ^{14}C were combusted to $^{14}CO_2$ and assayed by liquid scintillation counting. Radioactive mercurial compounds have also been used for the autoradiographic detection of reactive protein thiol groups on anuran hemoglobin chains.[148] In this case the protein was first denatured to produce maximum chain separation by carrying out the reaction in a 6 *M* urea medium. The protein (5–20 μg) in an acidic buffer solution was treated with [^{203}Hg]chlormerodrin and then subjected to starch-gel electrophoresis. Protein bands were detected with Nigrosine or Amido Schwarz B. Active bands containing bound ^{203}Hg were detected by autoradiography on X-ray film. It was shown that insulin and ribonuclease (which contained disulfide but not thiol groups) did not bind the mercuric compound, whereas with other proteins there was a general correlation between the thiol group content and the autoradiographic density.

Another sensitive radioassay for thiol groups used uniformly labeled [^{35}S]tetraethylthiuram disulfide (TETD).[149] The primary products of the interaction between protein and reagent were stoichiometric amounts of the [^{35}S]diethyldithiocarbamate ion and a derived protein carrying mixed disulfide linkages, each of which consisted of a protein thiol sulfur atom linked to a [^{35}S]diethyldithiocarbamyl residue (eq 36; R = protein).



(36)

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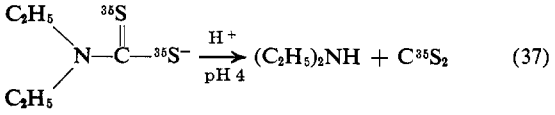
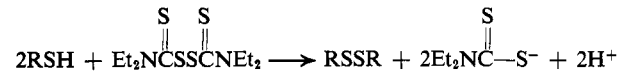
(145) H. Neurath, J. P. Greenstein, F. W. Putnam, and J. O. Erickson, *Chem. Rev.*, **34**, 157 (1944).
(146) V. G. Erwin and P. L. Pederson, *Anal. Biochem.*, **25**, 477 (1968).
(147) S. J. Leach, A. Meschers, and P. H. Springell, *ibid.*, **15**, 18 (1966).
(148) L. P. Stratton and E. Frieden, *Nature*, **216**, 932 (1968).
(149) A. H. Niems, D. S. Coffey, and L. Hellerman, *J. Biol. Chem.*, **241**, 3036 (1966).

The diethyl dithiocarbamate ion was separated and decomposed at pH 4 to give $^{35}CS_2$ (eq 37), which was trapped quantitatively in alkaline piperidine and its radioactivity measured by liquid scintillation counting. Alkylthiols do not react



(37)

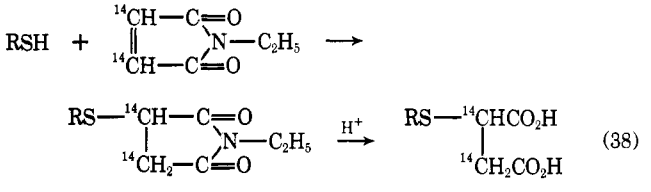
via eq 36 but instead give mostly alkyl disulfides.



However, an equivalent of dithiocarbamate ion is still formed. The method was applicable to 10-μg quantities of various proteins, and its precision was comparable to that of most existing methods. There was a possibility, however, of interference when cytochrome *c* or methemoglobin was present owing to oxidation of the dithiocarbamate.

The same reagent has also been used to study inhibition by TETD[150] of the enzymes, crystallized hog kidney flavoenzyme and D-amino acid oxidase. Evidence that such inhibition by TETD involved thiol–disulfide exchange reaction was as follows. One mole (taken to be 10^5 g) of D-amino acid oxidase contained 6–8 "immediately reactive" thiol groups out of a total of 12 such residues. The extent of inhibition of the oxidase was a linear function of the amount of TETD added; complete inhibition was attained with 6–8 molar equiv of TETD. Studies with [^{35}S]TETD showed the liberation of 6–8 molar equiv of [^{35}S]diethyldithiocarbamate ion and fixation to the protein of [^{35}S]diethyldithiocarbamyl residues. If the natural enzyme was denatured with a detergent such as sodium dodecyl sulfate, 12 molar equiv of DDC ion was produced directly.

The reagent which has received most attention, however, appears to be [^{14}C]-*N*-ethylmaleimide.[151–153] Treatment of peptides with an excess of labeled reagent was followed by complete hydrolysis of the addition products. [^{14}C]-*S*-Succinyl-L-cysteine, from flour (eq 38, RSH = L-cysteine), was isolated by paper chromatography and assayed for activity in a windowless gas-flow Geiger–Müller tube. To obviate



(38)

the necessity for a quantitative recovery of active *S*-succinyl-L-cysteine, a substoichiometric isotope dilution technique was employed using the addition product between inactive *N*-ethylmaleimide and glutathione as a carrier. A typical thiol content quoted was 1.32 μmol/g of flour, which was in good agreement with amperometric methods of determination. In the presence of urea, higher values were obtained because of greater chain separation. The quantitative nature of the method was demonstrated by analyzing standardized solutions of L-cysteine, reduced glutathione, and thiolated gelatin of known thiol content.[151]

Labeled *N*-ethylmaleimide has also been used to locate the available mercury binding sites in human hemoglobin.[154,155] This material is known to consist of four polypeptide chains of two types, α and β, and the two thiol groups per molecule should be either on the α or β chains, but not both. The question of which type of chain carried the thiol groups was answered by labeling with [^{14}C]-*N*-ethylmaleimide and determining the specific activities of the separated chains. The reagent was also used to study the role of the thiol groups and the effect of *N*-ethylmaleimide on oxygen equilibration in hemoglobin.

Two reagents which have been used to improve the sensitivity of the alkylation reaction were [^{14}C]iodoacetamide, which was allowed to react with thiol groups in human macroglobulin,[156] and [2-^{14}C]iodoacetic acid.[157] The latter reagent was used in a study of human 19S immunoglobulin M to distinguish between inter and intra subunit bridges. Interchain disulfide bridges were selectively reduced with 5 mM dithiothreitol in aqueous solution and the liberated thiol groups alkylated with the isotopic reagent.

VII. Analysis of Amino Compounds

The general analytical chemistry of amines is well documented and will not be discussed further.[158] Because of their widespread occurrence in substances of biological origin, for instance, amino acids, peptides, proteins, and enzymes, the measurement of low concentrations of amino groups is particularly important. There are many general references[159–163] to the measurement of amino acids, either as mixtures in protein hydrolysates or singly, as derivatives during N-terminal amino or sequence determinations in peptide chains. The more common methods of separation and measurement include high voltage paper electrophoresis,[164] ion exchange chromatography,[165] microbiological assay,[166] and gas–liquid chromatography,[167] and, where colored derivatives are being formed, spectrophotometry.[168]

When N-terminal amino groups are being measured, as in sequence determinations, the derivative method is particularly useful. Sanger[169] has used 1-fluoro-2,4-dinitrobenzene (DNFB) for the determination of the N-terminal groups of insulin. This reagent has now become widely used not only for protein end-group analysis but also for the determination of amino acids in protein hydrolysates. Methods of preparation and chromatographic separation of DNP amino

(150) A. H. Niems, D. S. Coffey, and L. Hellerman, *J. Biol. Chem.*, **241**, 5941 (1966).
(151) C. C. Lee and E. R. Samuels, *Can. J. Chem.*, **42**, 164 (1964).
(152) C. C. Lee and T.-S. Lai, *ibid.*, **45**, 1015 (1967).
(153) C. C. Lee and T.-S. Lai, *Cereal Chem.*, **44**, 620 (1967).
(154) A. Riggs, *J. Biol. Chem.*, **236**, 1948 (1961).
(155) A. Riggs and M. Wells, *Fed. Proc.*, **19**, 78 (1960).
(156) F. Miller and H. Metzger, *J. Biol. Chem.*, **240**, 4740 (1965).
(157) D. Beale and A. Feinstein, *Biochem. J.*, **112**, 187 (1969).
(158) J. Zabicky, "The Chemistry of Functional Groups—Amines," S. Patai, Ed., Interscience, New York, N. Y., 1968.
(159) H. D. Springall, "The Structural Chemistry of Proteins," Academic Press, New York, N. Y., 1954.
(160) H. G. Khorana, *Quart. Rev., Chem. Soc.*, **6**, 340 (1952).
(161) H. D. Springall and H. D. Law, *ibid.*, **10**, 230 (1956).
(162) A. H. Cook and G. Harris, *Progr. Org. Chem.*, **4**, 140 (1958).
(163) H. Fraenkel-Conrat, J. I. Harris, and A. L. Levy, *Methods Biochem. Anal.*, **2**, 359 (1955).
(164) S. Blackburn, *ibid.*, **13**, 1 (1966).
(165) S. Jacobs, *ibid.*, **14**, 177 (1967).
(166) E. F. Gale, *ibid.*, **4**, 285 (1957).
(167) B. Winstein, *ibid.*, **14**, 203 (1967).
(168) A. L. Levy, *Nature*, **174**, 127 (1954).
(169) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

acids have been reviewed,[170] while other workers have described procedures for their titrimetric,[171] colorimetric,[169] and spectrophotometric[168] estimation. The disadvantage of these methods lay in the fact that the terminal DNP–amino acid suffered partial destruction under the conditions of hydrolysis necessary to cleave them from DNP–peptides or proteins, and it was difficult to correct for this loss in nonisotopic experiments.

Although the DNP method is still widely used for end-group analysis, it is slowly being superseded by stepwise degradation methods such as the phenyl isothiocyanate procedure of Edman.[172] Phenyl isothiocyanate was coupled to the N-terminal amino acid to form the *N*-phenylthiocarbamyl derivative which was then selectively removed to yield the cyclic phenylthiohydantoin (PTH) of the terminal amino acid and a peptide chain having one amino acid less. These PTH derivatives could be estimated from ultraviolet absorption over the range 260–275 mμ, after suitable chromatographic separation. In early studies of the phenyl isothiocyanate method derivatives of serine, theorine, and cystine were shown to readily undergo decomposition,[173] although more suitable conditions have since been developed for their formation and isolation. The most sensitive of these methods was capable of measuring 0.1 μmol of an amino acid. However, with isotopically labeled reagents coupled with isotope dilution analysis, it was possible to increase the sensitivity by a factor of at least 10,000.

There are numerous early references in the literature[88–87,174–179] on the use of [^{131}I]-*p*-iodophenylsulfonyl chloride ([^{131}I]pipsyl chloride) for the determination of protein amino end groups and mixtures of amino acids in protein hydrolysates. Most of these methods employed a double isotope derivative technique in which the amino acid to be determined was quantitatively converted to its labeled pipsyl derivative (eq 39)

$$p\text{-}{}^{131}IC_6H_4SO_2Cl + H_2N{-}\underset{\displaystyle CO_2H}{\overset{\displaystyle |}{C}}H{-}R \longrightarrow p\text{-}{}^{131}IC_6H_4SO_2NH{-}\underset{\displaystyle CO_2H}{\overset{\displaystyle |}{C}}H{-}R \quad (39)$$

The labeled derivative could, at this stage, be isolated and purified by one or more of the available chromatographic procedures, but, because losses inevitably occurred during purification, a known amount of a second indicator pipsyl derivative of the same amino acid, labeled with a different isotope (usually ^{35}S), was added to the system prior to purification.

Keston, Udenfriend, and Cannan[88] were pioneers of the use of [^{131}I]pipsyl chloride for amino acid determinations. In the early work a large excess of unlabeled carrier pipsyl derivative of the same amino acid (or acids) was added to the reaction mixture prior to isolation and purification of labeled derivative. Thus, isolation was no longer quantitative, and it was only necessary to isolate sufficient material to enable its activity to be accurately determined. They[84] also examined separation of various [^{131}I]-*p*-iodophenylsulfonyl derivatives using ascending paper chromatography. Derivatives were prepared from 1-mg samples of various amino acids, indicator [^{35}S]-*p*-iodophenylsulfonyl derivatives were added, and aliquots equivalent to 1–7 μg were chromatographed on paper. From trial analyses carried out on glutamic acid, serine, glycine, and alanine, they obtained recoveries of 104, 91, 100, and 100%, respectively. Analysis of natural silk by this method showed 41% of the nitrogen to be present in glycine and 29% in alanine.

During the estimation of glycine, alanine, and proline in protein hydrolysates, it was found[35] that, although the reaction with [^{131}I]pipsyl chloride proceeded smoothly at 100°, small quantities of what were believed to be dipipsyl derivatives were formed from alanine and isoleucine but not from glycine or proline. Under appropriate conditions this side reaction could be increased up to 90% but not further. It was minimized by repeating the reaction with pipsyl chloride three times, each time removing the monopipsyl derivative of which totals of 98–100% were obtained. Isolation of the individual pipsyl derivatives after the addition of carriers was studied by crystallization, reprecipitation, and counter-current distribution between two solvents. The results of amino acid assays for β-lactoglobulin and crystalline aldolase were quoted. The recoveries of added amounts of glycine, alanine, and proline, from a mixture of 12 known synthetic amino acids, was also given.

In the estimation of glutamic acid, aspartic acid, hydroxyproline, serine, and threonine in 0.2–1.0 mg of hydrolyzed protein, addition of [^{35}S]pipsyl indicators and subsequent paper chromatographic separation were used.[36] Autoradiograms were used to locate bands, and the constancy of ^{131}I to ^{35}S ratios in successive portions of the bands provided a test of the validity of the analysis. A slight modification of this method was reported in a determination of glycine from a mixture of amino acids. [^{14}C]Glycine was added to the unknown mixture before preparation of the derivative with pipsyl chloride, thus increasing the accuracy of the method even more.[36] Analysis of proline, valine, methionine, and phenylalanine at the microgram level used [^{131}I]- and [^{35}S]pipsyl derivatives.[174] A made up mixture of 18 amino acids in the typical proportions found in proteins was analyzed using aliquots containing 0.2–2.0 μmol of each. Recovery of the four amino acids studied was shown to be satisfactory. It was also shown that the protein amino end-group derivatives formed from [^{131}I]pipsyl chloride were resistant to conditions which hydrolyzed the rest of the protein.[175,176] The amounts of *N*-pipsyllysine remaining after 2 and 16 hr of acid hydrolysis were 98 and 89%, respectively. Similar figures were also given for pipsylphenylalanine and pipsylglycine although the longer term stability of the later derivative was not as great (68% remaining after 16 hr). Addition of the ^{35}S-indicator derivative before hydrolysis to the constituent amino acids, however, provided automatic correction for partial destruction of the end group during hydrolysis. This method of protein amino end-group analysis resembles that using 2,4-dinitrofluorobenzene (DNFB),[169] but the pipsyl derivatives were more resistant to hydrolysis. Thus, the pipsyl method gave good results with proline in contrast with

(170) G. Biserte, J. W. Hollerman, J. Hollerman-Dehove, and P. Sautiere, *J. Chromatogr.*, **2**, 225 (1959).
(171) G. L. Mills, *Biochem. J.*, **50**, 707 (1952).
(172) P. Edman, *Acta Chem. Scand.*, **4**, 283 (1950).
(173) V. M. Ingram, *J. Chem. Soc.*, 3717 (1953).
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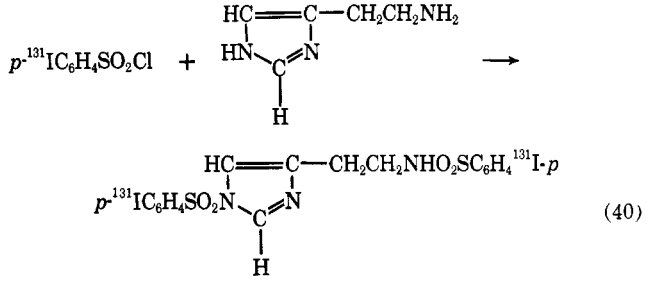
(175) S. Udenfriend and S. F. Velick, *ibid.*, **190**, 733 (1951).
(176) S. Udenfriend and S. F. Velick, *ibid.*, **191**, 233 (1951).
(177) R. W. Schayer, Y. Kobayashi, and R. L. Smiley, *ibid.*, **212**, 593 (1955).
(178) J. R. Fresco and R. C. Warner, *ibid.*, **215**, 751 (1955).
(179) S. Udenfriend, *ibid.*, **187**, 65 (1950).

DNFB where extensive hydrolytic breakdown occurred. However, analysis of bovine insulin samples weighing 200 μg showed one glycine and one phenylalanine amino end group per subunit of 12,000, whereas DNFB indicated two of each. Also, the pipsyl chloride method indicated two terminal valine units per molecule of hemoglobin, whereas Porter and Sanger[180] found 6. Since errors in the DNFB method would lead one to expect lower, rather than higher, results due to hydrolysis, it would appear that the pipsyl chloride did not in fact react with all the terminal amino groups under the conditions used.

Other workers have used the pipsyl isotope derivative method for determination of amino groups with success. Histamine was determined in various types of rat tissue by forming [^{131}I]dipipsylhistamine (eq 40).[177] Use of [^{14}C]-histamine and nonisotopic pipsyl chloride proved that yields were of the order 93 to 96%.



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The labeled derivative was mixed with inactive carrier dipipsylhistamine and repeatedly recrystallized to constant activity. Measured activities after four and five recrystallizations were quoted; in fact, constant activity was always achieved after the fourth recrystallization and very often after the third. Levels of histamine determined ranged from 1 μg/g of fresh tissue in rat liver to 33 μg in abdominal skin.

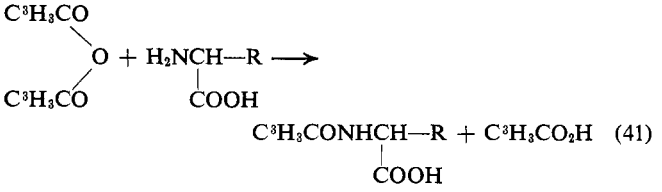
[^{131}I]- and [^{35}S]pipsyl derivatives were used to determine microgram quantities of uracil and thymine in nucleic acid mixtures.[178] This method had the advantage that it could be applied to hydrolysates of biological specimens from which the nucleic acids were not readily isolated. A consistent 94% conversion was obtained, and the monopipsyl derivatives were stable at room temperature below pH 10. In stronger alkali they were readily hydrolyzed to *p*-iodosulfonic acid and the free pyrimidines.

Finally, γ-aminobutyric acid was identified in extracts of mouse brain using [^{131}I]pipsyl chloride to form the derivative and [^{35}S]pipsylaminobutyric acid as indicator.[179] After separation of the derivatives by chromatography and location of the bands by autoradiography, the eluted bands were dried and counted with and without a 0.003-in. aluminum absorber using a thin window geiger counter. With the absorber in position, 40.2% of ^{131}I was transmitted and only 0.08% of the ^{35}S.

By comparison with later reagents, however, [^{131}I]pipsyl chloride had several disadvantages. The isotope used was a γ emitter of short half-life. Special radiation protection was therefore necessary when the isotope was handled, and correction was necessary for decay. Storage, handling, and dilution of the compound were complicated by the half-life of the isotope, the type of radiation, and the reactivity of the reagent toward water. It would, however, react quantitatively with the amino groups of amino acids and could be counted very simply in the presence of β emitters by use of filters in conjunction with a geiger tube. Extraction and purification of the derivatives was satisfactory, but they could only be located on chromatograms by scanning or autoradiography.

On the face of it, there are no objections to the use of [^{14}C]-pipsyl chloride since this overcomes most of the disadvantages associated with the ^{131}I-labeled reagent; however, no references to its use appear in the literature. This is probably because the energy of this β emitter is too close to that of the ^{35}S used in the indicator pipsyl derivatives to render them independently measurable. Tritium would be the obvious alternative.

A method of isotope derivative analysis employing [^{3}H]- and [^{14}C]-acetic anhydrides as reagents for the assay of amino acids has been described which permitted measurement of 10^{-5} μmol of an amino acid.[181] Use of *N*-acetyl derivatives, however, had a number of disadvantages which will be discussed later. [^{3}H]Acetic anhydride was used as



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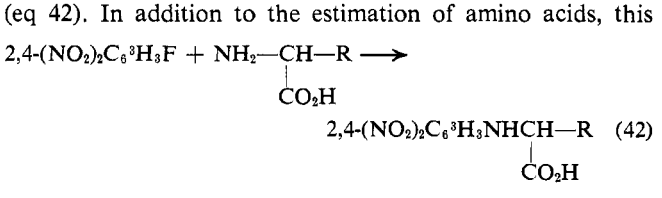
the acetylating agent (eq 41) and [^{14}C]-*N*-acetyl derivatives were added as indicators. The method was applied to synthetic mixtures of amino acids and to 72-hr hydrolysates obtained from 2 μg of two proteins of known composition. The ultimate practical sensitivity was given as 0.001–0.002 μg, although this would obviously depend on the specific activity of the acetylating agent used. Final recoveries of derivative after ion exchange and paper chromatographic separation varied between 10 and 25% according to the amino acid. The accuracy was normally in the region of 2–3%. Later this method was applied to the determination of thyroxine levels in human plasma.[182] The acetylthyroxine indicator was labeled with either ^{14}C or ^{131}I, and similar accuracies were claimed. Acetylation has also been used for the determination of 3-monoiodotyrosine and 3,5-diiodotyrosine.[183]

Because there were certain disadvantages associated with the use of labeled acetic anhydride for assay of amino acids, Beale and Whitehead[184,185] subsequently used [^{3}H]-1-fluoro-2,4-dinitrobenzene according to the method of Sanger[169] (eq 42). In addition to the estimation of amino acids, this



(42)

method was also suitable for N-terminal group determinations in 1 μg of protein, with an accuracy at least as good

(180) R. R. Porter and F. Sanger, *Biochem. J.*, **42**, 287 (1948).

(181) J. K. Whitehead, *ibid.*, **68**, 662 (1958).

(182) J. K. Whitehead and D. Beale, *Clin. Chim. Acta*, **4**, 710 (1959).

(183) D. Beale and J. K. Whitehead, *ibid.*, **5**, 150 (1960).

(184) D. Beale and J. K. Whitehead, "Tritium in the Physical and Biological Sciences," Vol. 1, International Atomic Energy Agency, Vienna, 1962, p 179.

(185) J. K. Whitehead, *Biochem. J.*, **80**, 35P (1961).

as that obtained by the established nonisotopic methods, which required as much as 1.0 mg of protein. [^{14}C]-2,4-Dinitrophenyl amino acid derivatives of known activity were added as indicators prior to separation by two-dimensional paper chromatography, and the ^{3}H and ^{14}C contents of each yellow spot were determined by combustion and gas counting. The analysis of a known mixture of eight amino acids indicated recoveries with an error of 4% for seven of them and 7.5% for phenylalanine. Analysis of a hydrolysate mixture equivalent to 0.90 μg of insulin gave values for the amino acid composition which were within 4.8% of the expected values. The recoveries of the [^{14}C]-2,4-dinitrophenyl amino acid indicator derivatives from a synthetic mixture of amino acids ranged from 15 to 52%. Various N-terminal group analyses for insulin and ribonuclease were also discussed. The method was also used[184] for the analysis of C-terminal groups of proteins using the hydrazinolysis technique.[186] When proteins were heated with anhydrous hydrazine, all but the C-terminal amino acids were converted into hydrazides (eq 43). The hydrazides were treated with benzaldehyde

$$NH_2CHRCONHCHR'CO_2H \xrightarrow{N_2H_4} NH_2CHRCONHNH_2 + NH_2CHR'CO_2H \quad (43)$$

to form the hydrazones which were then separated, leaving the C-terminal amino acids to be treated with [^{3}H]DNFB and isolated as previously described. This method gave results for the C-terminal amino acids of insulin and ribonuclease in agreement with the formulas, although hydrolysis of C-terminal asparagine gave a significant value for C-terminal aspartic acid in insulin.

A modification of the original method[184,185] was applied to urine and tissue.[187] Amino acids were allowed to react with [^{3}H]DNFB without prior isolation from the plasma, derivatives were extracted, and the [^{3}H]-2,4-dinitrophenyl-β-aminoisobutyric acid was separated by thin layer chromatography after carrier 2,4-dinitrophenyl-β-aminoisobutyric acid has been added to all the material at the origin of the chromatogram. The accuracy of the method was quoted as 10–15% but could obviously be improved by use of a second isotope as indicator, which would obviate the need for internal standards for calibration.

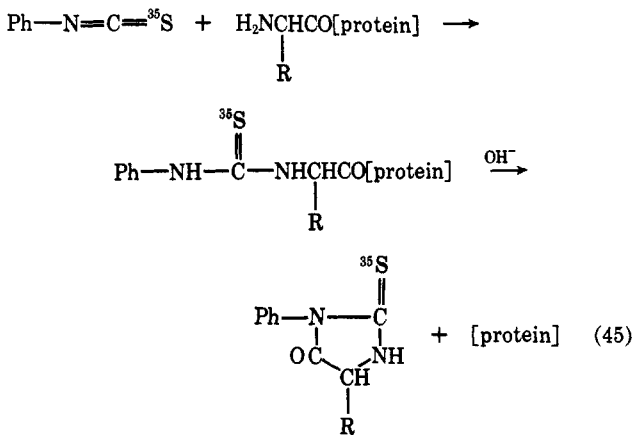
Dinitrophenyl derivative analysis had greater accuracy than did acetyl derivative analysis, because, with the use of reagents of similar equivalent activity, higher recoveries were obtained in the former method. Dinitrophenyl derivative analysis had several other advantages over the acetyl method. End-group analysis as well as amino acid analysis could be achieved. Chromatographic resolution was better and more reproducible, while the chromatogram spots were self-indicating. Dinitrophenylation and subsequent manipulation of labeled derivatives was much simpler and less time consuming, and in addition, the reagents and derivatives used in the dinitrophenyl method were much more stable to light and therefore required much less restandardization. It must be borne in mind, however, that under certain circumstances, for example, with proline, extensive hydrolytic breakdown of the derivative could occur when using this reagent.

[^{14}C]-2,4-DNFB has also been used in the calibration of a spectrophotometric method[130] for the determination of amino end groups in Nylon 66 (eq 44). Polymer samples were treated with [^{14}C]DNFB and the activity, after combustion to $^{14}CO_2$, compared with the absorbance at 440 mμ solutions containing labeled polymer. Results using this method were in excellent

$$2,4\text{-}(NO_2)_2{}^{14}C_6H_3F + H_2N(CH_2)_6NHCO\sim \longrightarrow 2,4\text{-}(NO_2)_2{}^{14}C_6H_3NH(CH_2)_6NHCO\sim \quad (44)$$

agreement with a conductometric titration procedure.[131] Titrimetric methods[131,188,189] for determining nylon amino end groups were often subject to interference from solvent residues (in fractionated material) and acidic or basic additives used in polymer modifications, and so the DNFB method had an immediate advantage. The reaction between polymer and DNFB must be carried out in a homogeneous solution since earlier workers[190] have demonstrated the inaccessibility of amino groups to DNFB in crystalline regions of the polymer when a heterogeneous system was used.

Several workers[191–196] have used phenyl [^{35}S]isothiocyanate for quantitative determination of N-terminal amino acids. The method was based on Edman's original technique[172] and involved coupling labeled phenyl isothiocyanate (PTC) to the terminal amino acid followed by cleavage of the [^{35}S]-phenylthiocarbamyl derivative from the peptide to produce the phenylthiohydantoin (PTH) (eq 45). The subsequent



isolation technique varied slightly with different workers. Callewaert and Vernon[194] added unlabeled carrier phenylthiohydantoin derivative and separated the mixture by paper chromatography. From 0.25 μmol of protein they obtained a value of 69,850 for the molecular weight of bovine serum albumin which was in very good agreement with the value of 69,000 obtained by ultracentrifugation. A thin layer chromatographic separation, without carrier addition, followed by direct measurement of activity using a radiochromatogram scanner was used successfully to determine 5 nmol of di-, tri-, and pentapeptide.[191–193] In another study N-terminal

(186) C. I. Nui and H. Fraenkel-Conrat, *J. Amer. Chem. Soc.*, 77, 5882 (1955).
(187) G. B. Gerber and J. Remy-Defraigne, *Anal. Biochem.*, 11, 396 (1965).
(188) P. W. Morgan and S. L. Kwolek, *J. Polym. Sci.*, 1, 1147 (1963).
(189) S. Wolf and B. Mobus, *Z. Anal. Chem.*, 186, 194 (1962).
(190) H. Zahn and P. Rathgeber, *Melliand Textilber.*, 34, 749 (1953).
(191) E. Cherbuliez, B. Bachler, M. C. Lebeau, A. R. Sussmann, and J. Rabinowitz, *Helv. Chim. Acta*, 43, 896 (1960).
(192) E. Cherbuliez, B. Bachler, and J. Rabinowitz, *ibid.*, 43, 1871 (1960).
(193) E. Cherbuliez, A. R. Sussmann, and J. Rabinowitz, *ibid.*, 44, 319 (1961).
(194) G. L. Callewaert and C. A. Vernon, *Biochem. J.*, 107, 728 (1968).
(195) W. G. Laver, *Virology*, 14, 499 (1961).
(196) W. G. Laver, *Biochim. Biophys. Acta*, 53, 469 (1961).

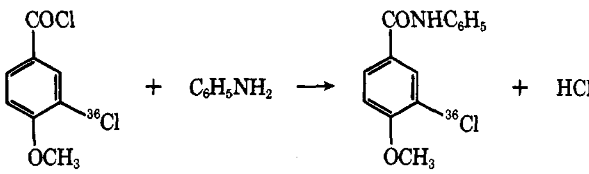
groups from 0.1 nmol of a number of known proteins were determined.[196] Carrier phenylthiohydantoin derivatives were used, and paper chromatographic separation was followed by either direct chromatogram scanning or elution and liquid scintillation counting. The quantitative studies indicated that yields of phenylthiohydantoin were between 30 and 40%.

[α-^{14}C]Benzyloxycarbonyl chloride in conjunction with a reverse isotope dilution procedure was successfully used for determining the optical purity of L-amino acids (eq 46).[197,198]

$$C_6H_4{}^{14}CH_2OCOCl + NH_2CH(CO_2H)R \longrightarrow C_6H_4{}^{14}CH_2OCONHCH(CO_2H)R \quad (46)$$

Absence of racemization during the acylation of nine monobasic acids was confirmed with this reagent, which was also used for the optical analysis of peptide hydrolysates. Thus, with a 7-mg sample of amino acid only 0.001% D-asparagine was found in the L isomer.

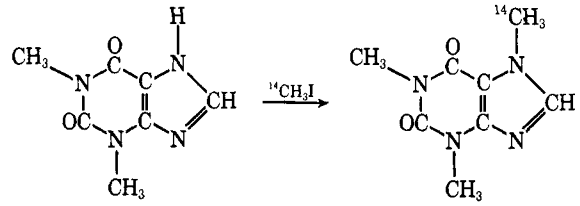
Another method for the determination of hydroxy and amino compounds used [^{36}Cl]-3-chloro-4-methoxybenzoyl chloride (eq 47).[86] The procedure differed from those described



(47)

earlier in that the inactive chloroanisoyl derivative was first formed, followed by the addition of a known amount of accurately standardized [^{36}Cl]chloroanisoyl derivative as indicator. The mixture was recrystallized from an appropriate solvent until the melting point differed by less than 1° from the pure derivative. Reaction with hydroxyl compounds was carried out under reflux conditions. However, with amines the mixture was maintained at room temperature; otherwise dichloroanisoylation occurred. An advantage of the method was that the only radioactive species present was the labeled derivative of the compound being analyzed. In procedures described earlier, labeled reagent could possibly react with impurities or compounds structurally related to the one being analyzed, in which case more rigorous isolation and purification procedures would be required. Percentage recoveries were quoted for various simple hydroxyl and amino compounds. These included phenol (94.3–101%), catechol (98.6–99.5%), methanol (99.3–101.8%), ethylene glycol (98.2–99.5%), aniline, (98.9–100.3%), and ethylenediamine (82.1–95.4%).

An isotope derivative dilution technique has been described for the determination of caffeine in drugs.[199] Theophylline was heated with labeled methyl iodide to give caffeine labeled in the 7-methyl position (eq 48). A known amount of labeled caffeine was added as indicator and the mixture isolated and counted after the addition of carrier. This procedure showed the presence of larger amounts of caffeine than were indicated by methods normally employed.



(48)

VIII. Analysis of Metal–Polymer Bonds

Labeled reagents have been used extensively for examination of growing polymer chains in anionic and cationic polymerization processes. The polymer chains were terminated by addition of an aliphatic alcohol to the system, forming carbon (polymer)–hydrogen bonds and a metal alkoxide in the case of an anionic process or the metal hydride and an ether-terminated polymer in a cationic system. By using an alcohol or other hydroxylic material suitably labeled in the hydroxyl hydrogen or alkyl carbon position, and locating the resulting activity, one has a sensitive means not only of actually measuring the number of polymer chains but also of determining the nature of the polymerization process.

[^{14}C]Carbon dioxide and [*hydroxyl*-^{3}H]acetic acid were used for the termination of methyl methacrylate polymerization initiated by 9-fluorenyllithium.[200] This particular initiator was used because of its intense uv absorption, enabling the number of initiating species per chain to be determined spectroscopically in conjunction with osmotic pressure measurements. They reasoned that if the polymerization did proceed through long-lived polymeric carbanions (they had already shown the chains were not rapidly self terminating), then it should be possible to determine their concentration by means of a reaction with the same reagents ordinarily used for quantitative analysis of simple low molecular weight organometallic compounds. Since the concentration of chain anions in a polymerizing system was likely to be low, the use of radioactive terminating agents was necessary to achieve maximum sensitivity. Both of the labeled reagents mentioned led to difficulties in their particular polymerizing system. The $^{14}CO_2$ reaction resulted in a disubstituted malonic half-acid ester structure at the chain end, which decarboxylated readily, resulting in loss of activity (eq 49). The use of tritium

$$Fl\text{∼}CH_2C(CO_2CH_3)(CH_3)^-Li^+ + {}^{14}CO_2 \rightleftharpoons Fl\text{∼}CH_2C(CO_2CH_3)(CH_3){-}CO_2^-\ Li^+ \quad (49)$$

labeling involved correction for a kinetic isotope effect which occurred in the breaking of O–H and O–^{3}H bonds during the reaction of acetic acid in a nonpolar solvent with the active chain ends (eq 50). Since the activity of tritiated poly-

$$Fl\text{∼}CH_2C(CO_2CH_3)(CH_3)^-\ Li^+ + {}^3HO_2CCH_3 \longrightarrow Fl\text{∼}CH_2C(CO_2CH_3)(CH_3){-}{}^3H + Li^+\ {}^-O_2CCH_3 \quad (50)$$

(197) W. R. Waterfield, *J. Chem. Soc.*, 2731 (1963).
(198) W. R. Waterfield, *ibid.*, 541 (1964).
(199) G. Arrani, R. Guiliano, and M. Zifferero, *Ann. Chim. (Rome)*, 47, 646 (1957).
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mers was stable with time, the latter method was the preferred one. When the polymerization reaction was terminated with acetic acid, any unreacted fluorenyllithium present was converted to radioactive fluorene. By use of standard isotope dilution techniques, the amount of unreacted initiator could be detected and was shown to be only 1.6% 5 sec after addition to the system. A kinetic isotope effect of 1.9 at −60° was reported. Hydroxyl tritiated methanol, ethanol, 1-propanol, and isobutyl alcohol have been used for determination of carbon–lithium bonds in polybutadienyllithium.[201] The specific activity of the labeled polymers was determined by reduction to a mixture of elemental tritium and tritiated methane followed by gas counting in an ionization chamber. The accuracy of the method was limited by the accuracy with which the isotope effect could be determined, an examination of their data indicating a possible variation from unity of approximately ±3% for this factor. Also, specific activity determinations of substrate and polymer, reproducible to ±1%, further decreased the certainty of absolute accuracy to an estimated value of ±5%. This radiochemical method for the determination of carbon–lithium bonds was subject to interference from other organolithium derivatives, which yielded nonvolatile compounds when treated with alcohols. In addition, care was necessary during isolation of the radioactive products to avoid nonquantitative recoveries due to the volatilization of relatively low molecular weight species.

Various other workers have also investigated the termination reaction of butadiene polymerization using labeled alcohols as quenching agents. One suggestion,[202] contrary to later workers, was that the polymerization yielding *cis*-1,4-polybutadiene was cationic in nature. Thus, termination by [^{14}C]methanol of chains initiated by triethyldialuminum chloride–cobalt octanoate resulted in incorporation of radioactivity into the polymer, whereas no incorporation occurred when [^{3}H]methanol was used. On the other hand, Cooper, Eaves, and Vaughan[203] suggested the polymerization to be anionic since termination by ^{14}C alcohols of active chains initiated by diethylaluminum chloride–cobalt naphthenate did not result in incorporation of activity into the polymer, whereas termination by ^{3}H-hydroxyl labeled alcohols did. They were not able to explain the presence of ^{14}C in polymers produced by Childers unless it was the result of his use of aluminum ethyl sesquichloride instead of aluminum diethyl chloride.

The same polymerizing system was studied with an aluminum diethyl chloride–cobalt diacetylacetonate catalyst.[204] Some runs were terminated by [^{14}C]butanol or methanol and others by tritiated methanol. The polymer was purified by repeated precipitation using methanol, as opposed to centrifugation and freeze drying. They showed that contamination of the polymer occurred in the reaction between growing polybutadiene chains and tritiated methanol in the presence of aluminum diethyl chloride. The occurrence of this contamination, and the fact that the tritium activity actually attributable to termination of growing chains was low, led them to suggest that termination by tritiated alcohols was unsuitable for the quantitative measurement of active centers. In addition, although reaction with tritiated alcohol destroyed the complex, it was not shown that destruction of the cobalt–carbon bond gave quantitative bonding of tritium to the polymer chain; it has been suggested that the bond could break homolytically as a consequence of destruction of the complex.

In the field of ethylene polymerization, hydroxyl-tritiated methanol was used to measure active centers initiated by titanium tetrachloride–alkylaluminum catalysts.[205] An isotope effect of 3.7 at 68° due to the tritium was found in this instance. When a bis(cyclopentadienyl)titanium dichloride–[^{14}C]-dimethylaluminum chloride catalyst system was used,[206] the concentration of the propagating metal–alkyl complex was determined by quenching aliquots of polymerizing mixtures with $^{131}I_2$. Prior quenching of polymer samples with butanol before the addition of $^{131}I_2$, either in the dark or under illumination, resulted in only background activity, thus indicating that incorporation of $^{131}I_2$ by reactions involving polymer unsaturation was negligible.

Tritiated methanol has also been used in studies of the kinetics of propylene polymerization initiated by titanium trichloride–diethyl aluminum chloride catalysts.[207] Aliquots of polymer slurry were released and quenched with tritiated methanol and then washed with excess methanol prior to repeated precipitation from xylene using the same reagent. Samples were finally counted by liquid scintillation after direct dissolution in fluor solution. The isotope effect of the quenching reaction was determined on a batch of polymer slurry by treating several portions of it with radioactive quench solution in the usual manner, while others were titrated with a more dilute solution of tritiated methanol in xylene and amine. The isotope effect, taken as the average value of the ratio of polymer count from the titrated and untitrated samples, was 1.3 ± 0.1%.

No significant isotope effect could be detected when [^{3}H]-butanol was employed for the termination at 50° of polymerizations of propylene initiated by various Ziegler catalysts.[208] Three catalysts were investigated, two of these being based on titanium trichloride and aluminum diethyl monochloride (H-catalyst), while the third consisted of titanium trichloride and triethylaluminum (N-catalyst). From the rates of propagation, the incorporated radioactivity, and the number-average molecular weight at different reaction times, it was shown that the macromolecules grew during almost the entire polymerization period with both types of catalyst, and there was, if any, only a limited chain transfer reaction. The rate of propagation was smaller than that of other polymerization reactions and the active centers of the catalysts were in part released during an initial phase in 1–1.5 hr, H-catalysts having a greater number of active sites than N-catalysts.

IX. Analysis of Free Radicals

Radiolabeled scavengers have been used by several workers in studies of free radical formation during irradiation of

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(202) B. Childers, *J. Amer. Chem. Soc.*, **85**, 229 (1963).
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(204) G. Natta, L. Porri, A. Carbonaro, and A. Greco, *ibid.*, **71**, 207 (1964).
(205) C. F. Feldman and E. Perry, *J. Polym. Sci.*, **46**, 217 (1960).
(206) J. C. W. Chien, *J. Amer. Chem. Soc.*, **81**, 86 (1959).
(207) E. Kohn, H. J. L. Schuurmans, J. V. Cavender, and R. A. Mendelson, *J. Polym. Sci.*, **58**, 681 (1962).
(208) G. Bier, W. Hoffmann, C. Lehmann, and G. Seydel, *Makromol. Chem.*, **58**, 1 (1962).

solid polymers[209] or radiolysis of liquid hydrocarbons.[210,211]

A doubly labeled scavenger, phenyl [^{3}H]-*sec*-butyl [^{35}S]-disulfide, was used for quantitative measurement of free radicals formed during irradiation of polyisobutene.[209] Traditional scavenging techniques relied on the spectrophotometric measurement of diphenylpicrylhydrazyl consumption or the determination of alkyl iodides formed by the addition of iodine to the system. In all these earlier cases, however, the hydrocarbons were mobile. Use of an isotopically labeled scavenger extended quantitative scavenging techniques to an exceedingly viscous hydrocarbon having a viscosity average molecular weight of about one million. After irradiation and purification, samples were assayed directly by liquid scintillation counting. For singly labeled compounds, the standard error of assay was ±3% and for doubly labeled compounds, about ±5%. A comparison of the results with those for mobile hydrocarbons showed that the high viscosity did not reduce the yield of radicals, but an increased concentration of scavenger was required to trap them.

A labeled radical scavenger was used in studies of the depolymerization of rubbers on cold mastication.[212] Theory postulated that mechanical rupture of the elastomer molecules occurred during the mastication process to give polymeric free radicals which either recombined to give an undegraded polymer, or under suitable circumstances reacted with a radical acceptor. The intervention of the radical acceptor thus consummated the chain rupture of the elastomer as evidenced by an increase in plasticity. Cold mastication of a butadiene–styrene copolymer under nitrogen and in the presence of 1,1′-dinaphthyl [^{35}S]disulfide resulted in incorporation of 1-naphthyl [^{35}S]sulfenyl groups in the degraded polymer molecules. The radiochemical estimate of the number of new chains formed during depolymerization was in agreement with the number of new chains formed as calculated from osmotic molecular weight data. A similar study of the ultrasonic degradation of poly(methyl methacrylate) and polystyrene used $^{131}I_2$ as the radical acceptor.[213]

A radiochemical technique has been developed to measure the yields of radical species produced in the radiolysis of liquid hydrocarbons.[210] Radioactively labeled ethyl radicals, formed during the irradiation by addition of hydrogen atoms to [^{14}C]ethylene, were found to scavenge radicals to an extent proportional to their relative concentrations. The yields of ^{14}C hydrocarbons measured the corresponding radical yields. The total activity in each component obtained from radiolysis of *n*-pentane was measured with a flow proportional counter connected to the effluent of a conventional gas chromatographic detector. This labeling technique had the advantage that the products of the scavenging reaction could be distinguished from the multitude of products ordinarily observed in the radiolysis of liquid hydrocarbons. It was suggested that the technique could also be used to prepare labeled hydrocarbons of specific activity approaching that of the [^{14}C]ethylene used.

The same workers[211] later used [^{14}C]methyl rather than -ethyl radicals as scavengers in similar studies of the radiolysis of saturated and unsaturated hydrocarbons. A further advantage of [^{14}C]methyl radicals was that in general there was only one disproportionation step (gain of a hydrogen atom) to consider, whereas with ethyl radicals both disproportionation steps (gain or loss of a hydrogen atom from the scavenging radical) had to be considered. [^{14}C]Methyl iodide was chosen as a source of the scavenger [^{14}C]methyl radicals since it was known that when a low concentration of methyl iodide was present during radiolysis in hydrocarbons such as *n*-pentane[214] and *n*-hexane,[215] methyl radicals were generated. A unique advantage in this case was that [^{14}C]-methyl iodide could be used in alkenes, aromatic systems, and other reactive solvents, whereas before this, no chemical scavenger was available for the determination of individual radical yields.

X. Analysis of Unsaturation

Several reagents have been developed for the measurement of olefinic unsaturation. [*carbonyl*-^{14}C]Maleic anhydride was a convenient reagent to measure the concentrations of various dienes and was used to study the competitive rates of Diels–Alder reactions by an isotope dilution technique.[216]

Chlorine-36 gas proved to be a very satisfactory reagent for micro determination of unsaturation in soluble polymers such as butyl rubber[217] and polyisobutene.[218] Unsaturations of between 0.1 and 0.01 mol % could be measured in 250-mg samples of polyisobutene using a simple vacuum technique to introduce the $^{36}Cl_2$ into polymer solutions. Unsaturation in copolymers of propylene oxide and unsaturated epoxides may be determined[219,220] in the presence of antioxidants by means of [^{14}C]methanol. The polymers were dissolved in ethylene dichloride and treated with an excess of [^{14}C]-methanol and mercuric acetate to yield the vicinal [*methoxy*-^{14}C]acetoxymercuric derivative of the double bond (eq 51). Excess methanol and solvent were removed and the radioactivity was determined by wet combustion to $^{14}CO_2$ which was assayed in an ionization chamber. With a sample size of

$$\mathrm{H(OCHCH_2)_nOCH_2CH{=}CH_2} + {}^{14}\mathrm{CH_3OH} + \mathrm{Hg(OAc)_2} \longrightarrow \mathrm{H(OCHCH_2)_nOCH_2CH(O{}^{14}CH_3)CH_2(HgOAc)} + \mathrm{HOAc} \quad (51)$$

about 30 mg it was possible to measure unsaturation in the range 0.5–1.5 mmol/g. Agreement was good between the radiochemical method and a more conventional titrimetric method, but, as with most of the analyses described in this review, the radiochemical method had the advantage of being applicable to micro amounts of substrate.

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(218) I. C. McNiell and R. McGuchan, *J. Polym. Sci., Part A-1*, **4**, 2051 (1966).
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(220) D. R. Campbell, *J. Appl. Polym. Sci.*, **14**, 847 (1970).

XI. Miscellaneous Analyses

A method of analysis not strictly involving reaction with a functional group was used for the determination of estrone, estriol, and estradiol as their 2,4-dibromo derivatives.[221] Samples were brominated with $^{82}Br_2$ and chromatographed after the addition of appropriate unlabeled carrier derivatives. The fractions were assayed by absorption at 291 mμ, and further amounts of carrier were added to each peak prior to recrystallization to constant activity. Overall recoveries at the 10-ng level were given as 80% for urine and 95% for plasma, with a sensitivity of 1 ng and an accuracy of ±3%. The disadvantages of this particular isotope are the short half-life (36 hr) and the fact that it is a hard γ emitter, necessitating extensive protective shielding during the analytical procedure.

Tetrazolium salts labeled with ^{14}C have been used by several workers in a simple isotope derivative procedure for the determination of corticosteroids in plasma. In one study paper chromatography was used to separate the individual steroids which were then treated with a standard solution of [^{14}C]tetrazolium salt, either before or after elution of the spots.[222] Dried, purified samples were planchette counted in an argon–amyl alcohol flow counter. The sensitivity was given as 0.1–0.01 μg depending on the reagent used, with errors of 10 and 5%, respectively for samples of 0.01 and 0.05 μg. A slightly different method involved acetylation of corticosterone and aldosterone before treatment with [^{14}C]tetrazolium blue.[223] Excess reagent was washed off, the paper chromatogram was dried and cut out, and the spots were eluted into a liquid scintillation counting fluor solution for measurement of [^{14}C]formazan activity. Although the method was less sensitive than a double isotope procedure, a sensitivity ten times greater than that obtained using unlabeled tetrazolium blue was claimed.

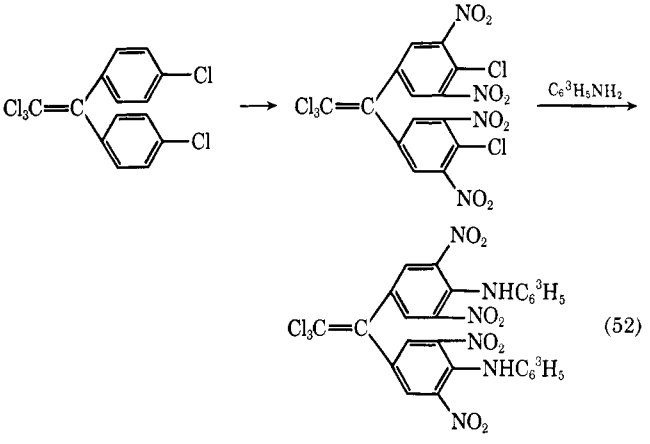
A double isotope derivative technique for the determination of strychnine in blood used [^{3}H]methyl iodide as the reagent and the corresponding [^{14}C]strychnine methiodide as the indicator derivative.[224] After derivative formation, the methiodide was chromatographed on paper and the spot, located by spraying a strip of the paper where unlabeled derivative had been applied, was then cut out and counted in a liquid scintillation counter. Correction was made for the counting efficiency by counting a standard sample of [^{14}C]strychnine methiodide which had been run on the same paper chromatogram.

A photochemical method was used for the determination of submicrogram amounts of flavines.[225] [*carboxy*-^{14}C]-1-Naphthylacetic acid was decomposed by flavines on exposure to visible light (1 hr under 340-mμ line) with evolution of $^{14}CO_2$, which was measured and related to the amount of flavine initially present. The method was applicable to the determination of flavines in the concentration range 10^{-11} to 10^{-9} mol.

For the determination of nucleotide sequences in molecules of soluble, ribosomal, and messenger RNA Augusti-Tocco and Brown[226] used [^{14}C]-4-methyl-*N*-cyclohexyl-*N'*-(β-(4-methylmorphino)ethyl)carbodiimide iodide. In the reaction of this compound with synthetic polynucleotides and nucleic acids, preference was shown for the bases of non-H-bonded uridylic, thymidylic, and guanylic residues in RNA and denatured DNA. The radioactivities of the samples were determined with a gas flow counter and the moles of reagent bound per mole of nucleotide were calculated from the specific radioactivity of the reagent and the amounts of polynucleotide or nucleic acid added to the reaction mixtures. It was concluded from the reactions with DNA and synthetic polynucleotides that the nucleotide residues in sRNA that reacted with the labeled reagent were mainly in the randomly coiled regions of the molecule. These regions could therefore be labeled with this reagent which rendered them identifiable in digests of sRNA made with specific R bases.

[^{131}I]Iodine monochloride was used as a reagent to form the [^{131}I]-3,5-diiodo derivative of salicyclic acid in an inverse isotope dilution analysis of this compound.[227] The procedure was also applied for the assay of acetylsalicylic acid after quantitative hydrolysis to the hydroxy acid.

Various double isotope derivative procedures have been used for the determination of microgram quantities of pesticide residues in tissues and foodstuffs.[228] The work involved a study of possible methods for the determination of DDT, DDE, Dieldrin, Systox, and Diazinon. DDT and DDE were tetranitrated after the addition of similar ^{14}C indicator compounds, and then the dianilides were formed using [^{3}H]-aniline (eq 52). Derivatives were purified to a constant isotopic



(52)

ratio by paper chromatography and then counted. Determinations at levels down to 1 μg were described, but no details were given of the application of the procedure to materials of biological origin.

Dieldrin was determined using [^{3}H]acetic anhydride as the reagent which, in the presence of hydrobromic acid, led to the formation of the 6-acetoxy-7-bromo derivative. These workers made an extensive investigation of the chromatographic purification of the dieldrin derivative but were unable to isolate a material of the required radiochemical purity. Multiple recrystallization of the derivative from 70 μg of dieldrin in the presence of 50 mg of carrier gave a product of the required purity. The same group of workers proposed

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to determine both the thiol and thiono isomers of Systox as the [^{3}H]-2,4-dinitrobenzoyl derivative following hydrolytic cleavage to β-mercaptodiethyl sulfide and β-hydroxydiethyl sulfide, respectively. They prepared [^{14}C]thionosystox but were unable to complete the analysis because of nonavailability of the tritiated reagent. It was also proposed that diazonin should be cleaved to the hydroxypyrimidine and a derivative prepared using [^{3}H]acetic anhydride. This, however, proved impracticable.

^{14}C-Labeled ethanol was used to measure the number of acid chloride side chains formed during the cross-linking of cellulose acetate monofilaments by bis acid chlorides.[229] The number of combined ^{14}C-ethoxy residues giving a direct measure of the number of side chains present (eq 53, R = polymer).

$$\mathrm{RCOCl} + {}^{14}\mathrm{CH_3CH_2OH} \longrightarrow \mathrm{RCOOCH_2{}^{14}CH_3} + \mathrm{HCl} \quad (53)$$

[^{14}C]-*N*-Ethylmaleimide, the reagent developed for measurement of thiol groups, has also been used to determine sulfite ions in biological fluids.[230] Urine containing less than 40 nmol of bisulfite was incubated with 40 nmol of [^{14}C]-*N*-ethylmaleimide at pH 7.4, at room temperature under nitrogen for 30 min. The adduct was purified by paper chromatography, and strips of the chromatogram were cut out and immersed in a liquid scintillation fluor for assay.

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