THE GIRARD REAGENTS

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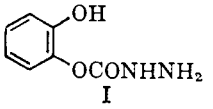
I. FORMULAS OF THE REAGENTS

The first reagents used for preparing soluble derivatives of carbonyl compounds, apart from sodium bisulfite, were the mono-N-aminocarbamates introduced by Einhorn in 1898, initially the hydrazine of the monocarbamate of pyrocatechol (I) (47) and later the corresponding derivatives of resorcinol and hydroquinone (48). However only aldehydes react readily with these reagents and the resulting hydrazones are difficult to hydrolyze. Aryl methyl ketones react slowly in the presence of acetic acid and zinc chloride. Thiele, in 1898, showed that salts of aminoguanidine derivatives, the nitrates and picrates excepted, were soluble but that these derivatives were also resistant to hydrolysis (178, 179). *p*-Hydrazinobenzenesulfonic acid (II) first introduced in 1902 (15), forms hydrazones with aliphatic and isocyclic ketones but not with aldehydes (181), and has been used to separate ketones from aldehydes (see section X). However the reagents that have found most general use are the quaternary ammonium acetyl hydrazides (III). The first of these to be introduced and those still most widely used, were



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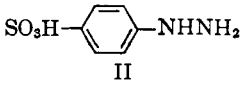

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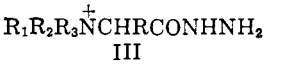

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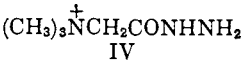

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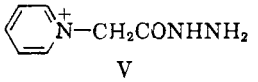

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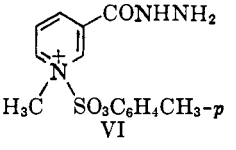


VI

trimethylammonium acetyl hydrazide chloride (betaine hydrazide chloride) (IV; III, $R_1 = R_2 = R_3 = CH_3$; R = H) and pyridinium acetyl hydrazide chloride (V; III, R_1, R_2, R_3 = pyridinium ring, R = H) introduced by Girard and Sandulesco in 1934 (67, 68). These are known as the Girard-T and -P reagents, respectively, and are widely used in preparing water-soluble derivatives, the Girard-T and -P hydrazones, of both aldehydes and ketones. The allied quaternary salt, N-methyl-β-carbohydrazide pyridinium *p*-toluenesulfonate (VI; III, $R_1 = CH_3$; R_2, R_3, and R part of the pyridinium ring) has also found limited use (1) and has the advantage over the Girard reagents since it forms solid derivatives with aliphatic compounds. The *o*- and *p*-carboxyphenylhydrazines (VII) (2,

183) and carboxymethylhydrazine (VIII) and carboxymethoxylamine (IX) (2, 18) have found limited use in preparing water-soluble carbonyl derivatives (see section X). An optically active Girard reagent, dimethyl-(α-methyl-β-phenylethyl)ammonium acetyl hydrazide chloride ($PhCH_2CHCH_3{}^+N(CH_3)_2CH_2CONHNH_2$; III, R = R_2 = CH_3; R_3 = $PhCH_2CHCH_3$; R = H) has also been prepared (39).

$CO_2HPhNHNH_2$ VII $CO_2HCH_2NHNH_2$ VIII $CO_2HCH_2ONH_2$ IX

This review will be principally restricted to the properties and reactions of the Girard-T and -P reagents. Brief reviews covering these reagents have appeared in 1939 (130), 1948 (131), and 1954 (36).

II. Preparation and Properties of the Reagents

The reported preparations of the Girard-T and -P reagents (36, 66, 130) follow essentially the original directions of Girard and Sandulesco (70), which consist in condensing ethyl monochloroacetate with either trimethylamine or pyridine and then refluxing the resulting quaternary ammonium ester with hydrazine or its hydrate. The optically active Girard reagent mentioned above was similarly prepared from active dimethyl-(α-methyl-β-phenyl)-ethylamine (39). The presence of the diacetyl hydrazides in the Girard reagents is avoided by using an excess of hydrazine (130), when the yields are 90%.

The Girard-T reagent, m.p. 192° with decomposition, is very soluble in water, soluble in cold ethanol (1 part in 150), soluble in methanol, 2-propanol (201), glycerol and ethylene glycol, very soluble in acetic acid, but insoluble in nonhydroxylic organic solvents (70, 172). It is very hygroscopic and develops a disagreeable odor on standing, but can be purified by washing the crystals with ethanol (130). It has been recommended that the reagent be preserved by storing with 15–20% ethanol and using the reagent as such while taking into account the weight of ethanol (130).

The Girard-P reagent, decomposing at 200° before melting, is less soluble in polar solvents and is best recrystallized from methanol. It is not hygroscopic and can be stored in air with no special precautions (130).

III. Pharmacological Properties

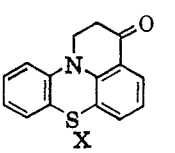
Girard-T and -P reagents, being very soluble in water, have been tested for their pharmacological activity. Both compounds inhibit the action of histidine decarboxylase in kidney, liver, and duodenal tissue of rodents (196), and act as catalase poisons, thus having a potential use in relation to protection against radiation (20). However semicarbazide hydrochloride and methoxyhydroxylamine hydrochloride also show similar activity and hydroxylamine and sodium azide are more specific catalase poisons. The Girard reagents have been tested for acetylcholine activity (86), Girard-T against *tubercle bacilli*, toward which it shows a 1.0–0.5 mg.% inhibitory action (123) and Girard-P for tuberculostatic activity both *in vitro* and *in vivo* (in the mouse corneal test) (11). Girard-P also acts as an inhibitor on aryl-sulfatase of *Alcaligens metalcaligens*, showing 25% inhibition at 2.5% (w./v.) (40) and being much less active than other carbonyl reagents such as semicarbazide, hydrazine, and hydroxylamine.

A number of Girard hydrazones of drugs or potential drugs have been prepared, to render these more water soluble. Thus the Girard-T hydrazone of 5-nitro-2-furaldehyde has been found to suppress experimental *Trypanosoma cruzi* infection in mice (129), although the same compound has little fungistatic activity against a number of *Trichophytons*, *Microsporon lanosum*, and *Epidermophyton albicans* (195). This same compound, the corresponding Girard-P hydrazone and the Girard-P hydrazone of 5-nitrofuryl caprylate have been prepared as chemotherapeutic agents (194). The Girard-T derivatives of 2-thiophene aldehyde, 5-methyl, 5-nitro, and 5-bromo-2-thiophene aldehyde and the Girard-P derivatives of 2-thiophene aldehyde and 5-nitro and 5-bromo-2-thiophene aldehyde have been tested as pesticides (77, 78).

The Girard-P hydrazones of *p*-nitrobenzaldehyde and *p*-acetylbenzaldehyde showed no tuberculostatic activity *in vitro* (117), although hydrazides generally show such activity (208).

The Girard-T hydrazone of 2-methyl-1,4-naphthoquinone has been tested for vitamin K activity (17), but the corresponding Girard-P compound was ineffective at the 12 μg. level in the chick-assay coagulation test (23). The Girard-T hydrazones of *o*-quinones of dihydroindoles, such as *l*-andrenochrome and epinochrome (167, 168) have been tested for antipressor effect in animals.

A number of Girard-P derivatives of phenanthridine aldehydes have been prepared and tested against *Streptococcus pyrogenes* (161). The Girard-T and -P hydrazones of phenanthridine-9-aldehyde and its 7-carbethoxyamino-2,7-dicarbethoxyamino, and 2-nitro derivatives have high anti-streptoccal activity *in vitro*, the carbethoxyamino compounds being less potent, although all these compounds showed no trypanocidal activity (31). 2,3-Dihydro-3-keto-1H-pyrido[3.2.1-kl]-phenothiazine (X) forms a Girard-P derivative which



has anthelmintic properties (109), as well as an insoluble azine (110) (see section IVE).

IV. SEPARATION OF KETONES FROM NON-KETONIC COMPOUNDS

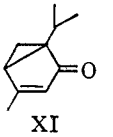
A. General Procedure

The Girard-T and -P hydrazines commonly are formed in 10% acetic acid in ethanol or methanol using a large excess of the reagent as a 5–10% solution, and refluxing for 20–30 minutes, or up to 1 hour (70) or even 12 hours (131) in the case of less reactive ketones. An acid ion-exchange resin (Amberlite IRC 50) also has been used as acid catalyst (180). The solutions of the Girard hydrazones are stable in nearly neutral solution at pH 6.5–7, but are readily hydrolyzed in acid medium (70). The cooled solution is usually treated with aqueous sodium hydroxide to neutralize some $^9/_{10}$ of the acetic acid, being titrated to bromothymol (70, 131) if necessary. The partially neutralized solution is then diluted with water to 10–20% volume of the ethanol and extracted several times with a non-hydroxylic solvent such as diethyl ether (70), diisopropyl ether (131), or methylene chloride (157, 158) to remove unreacted nonketonic material. The extracted solution is then hydrolyzed to regenerate the ketones. Hydrolysis is rapid at room temperature at pH < 6 and can be carried out by adding acetic acid or oxalic acid, although hydrochloric or sulfuric acid are usually added to 0.1–1 *N* solutions. The time needed varies from 1 minute to 1 hour and the solution is often heated briefly to complete the hydrolysis. The ketone fraction is then extracted with ether, although this procedure often leads to the formation of emulsions. These can be broken by centrifugation or by adding ethanol to 20% volume of the aqueous solution (131), but are best avoided by using ethylene glycol (70) instead of ethanol or methanol in the formation of the Girard hydrazone, which is then carried out by heating on a steam-bath. In this case the acidified solution is finally extracted with dry ether. Girard and Sandulesco (70) also recommended adding ethylene glycol to the ethanol solution and then distilling off the ethanol before extracting with ether.

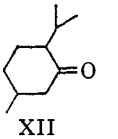
The Girard reagents have found considerable use in extracting ketones from nonketonic substances, principally from essential oils and mixtures of steroids and offer the advantage that they form solid precipitates with certain inorganic reagents (see section VI). Their reaction with aldehydes shows some differences with respect to those of ketones, and these and other aspects of their reactions are discussed in the following sections.

B. Essential Oils and General Uses

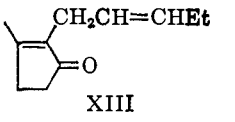
Essential oils are usually complex mixtures of hydrocarbons, carbinols, esters and ketones, and the Girard reagents frequently have been used to separate the ketones from the other components (36, 116, 124, 130, 172). Thus umbellulone (XI) has been separated from Kenya cypress oil in which it occurs as 8.6% of the fraction b.p. 130–200° (165), and separation with Girard-T reagent is much more efficient than with semicarbazide (164). Isomenthone (XII) has been isolated in the same manner from Algerian geranium oil (5, 142, 143) and isomenthone, a $C_{15}H_{22}O_2$ ketone, three other unidentified ketones, and an aldehyde obtained from La Reunion geranium oil (163). The ketones



XI



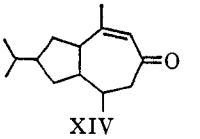
XII



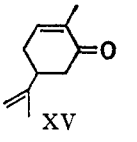
XIII

of a lavender oil have been similarly separated (88, 142, 143), including compounds having the odor of methylheptenone and carvone (155) and a C_{10} ketone which gave a Girard-T hydrazone which was not hydrolyzed by cold hydrochloric acid (166). A new $C_{13}H_{22}O$ ketone, parmone, of unknown structure was extracted from violet oil (147) and jasmone (XIII) and an unidentified $C_{12}H_{16}O_3$ ketolactone, an isomer of calythrone, from jasmin "concrète" (125).

The oil of case (essential oil of *Juniperus oxydrusce L.*) was shown to contain 3–4% aldehyde and ketone, including vetivone (XIV) and three unidentified ketones (170), whereas 33% carbonyl compounds were isolated from verbena oil, being principally methylheptenone, carvone (XV), and furfural and traces of α- and β-citral (89, 90). Bourbon vetiver essence gave 13.6% crude ketone, of which 78.9% was vetivone (XIV), and a corresponding java oil afforded 13% ketone on extraction with Girard-P reagent (154), although it has been reported that this reagent is unsatisfactory for the isolation of vetivones from vetiver oil, semicarbazide being more suitable (132).



XIV



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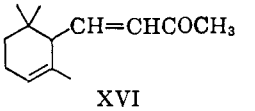
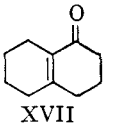


XVI



XVII

In the analysis of benzylidene-acetone in revulsive a sample of 50 g. containing about 0.2 g. of the substances was treated with a slight excess of Girard-T reagent, the terpenes extracted with ether and the hydrazone hydrolyzed for 1 to 2 hr. with 0.5 to 1 *N* hydrochloric acid giving the benzylidene-acetone in 70–75% yield (33).

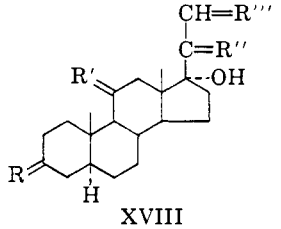
The Girard reagents also have found some more general uses in separating ketones from other compounds. Thus the unreacted α-ionone (XVI) was removed from

its reduction product α-ionol by extraction with Girard-T reagent (94) and β-ionone (the conjugate isomer of XVI) was separated from its alcohol using the Girard-P reagent (95). Recently $\Delta^{9(10)}$-1-octalone (XVII) was purified with Girard-T reagent, the separation being more nearly complete than with hydroxylamine hydrochloride since the product showed a smaller infrared band of an unconjugate or saturated ketone (24). The removal of ketonic impurities has assisted in the purification of vitamin E (42).

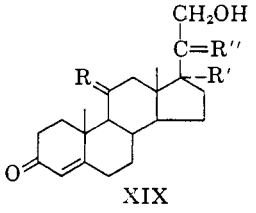
Girard and Sandulesco report (70) a method of freeing methanol from acetone by adding 5% acetic acid and Girard-P reagent, heating 1 hr., and distilling the methanol, which is redistilled from sodium to eliminate traces of acetic acid.

C. Steroids

The Girard reagents were first used (56) by their discoverers (70) to separate estrone from urinary extracts (67, 68, 69), and a few years later by Reichstein with considerable success to separate the adrenal cortex steroids into his substances A (allopregnane-3β,11β,17α,20β,21-pentol; XVIII, R = R′ = R″ = R‴ = H, OH), C (allopregnane-3α,11β,17α,21-tetrol-20-one; XVIII, R = R′ = R‴ = H, OH, R″ = O), D (allopregnane-3β,17α,21-tetrol-11,20-dione, XVIII, R = R‴ = H, OH, R′ = R″ = O), E (pregn-4-ene-11β,-17α,20β,21-tetrol-3-one, XIX, R = R′ = R″ = H. OH) and F (impure cortisone) (140) and later compounds Fa (pure cortisone, XIX, R = R″ = O, R′ = OH) H (corticosterone, XIX, R = H, OH, R′ = H, R″ = O) and J (allopregnane-3β,17α,20β-



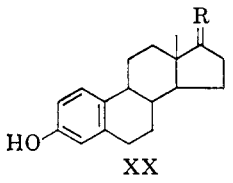
XVIII



XIX

triol, XVIII, R = R′ = R″ = H, OH, R‴ = H_2) (141). He introduced the use of selective hydrolysis (*cf.* section VIIB) and reported that α,β-unsaturated ketones (Δ^4-3-ketosteroids) react more readily than saturated ketones, but require a higher concentration of acid for hydrolysis (141).

The standard Girard procedure has since been used in a number of separations of ketonic and nonketonic fractions from mixtures of steroids such as urinary steroids (115, 133), thymus corticosteroids (139) and the steroids in human sperm (38), the neutral (nonphenolic) 17-ketosteroids in urinary extracts (7), the incubation products of desoxycorticosterone (XIX, R = H_2, R′ = H, R″ = O) (177) and the isolation of estrone (XX, R = O) from late pregnancy cow urine, from which an unidentified more hydrophilic estrogen was also isolated (134). Allopregnane-3-ol-20-one (XXI)



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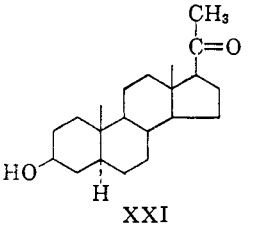
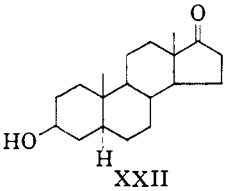


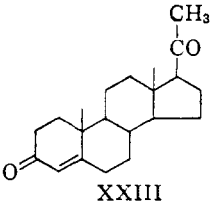
XXI

has been isolated from the ketonic fraction of human pregnancy urine (112) and 17-hydroxycorticosterone (XIX, R = H.OH, R′ = OH, R″ = A) and 17-hydroxyl-11-dehydrocorticosterone (cortisone, substance F) isolated from the adrenal corticol hormones in urine (115). The Girard-T separation of the adrenal-corticol steroids of a case of congenital hyperplasia and systemic hypertension assisted in the isolation of Δ^4-pregnene-17α,21-diol-3,20-dione, pregnane-3α,17α,21-triol-20-one, androstane-3α-ol-17-one (XXII), etiocholane-3α-ol-17-one (XXII, ring A/B *cis*), pregnane-3α,20α-diol and pregnane-3α,17α,20α-triol in the blood and pregnane-



XXII

3α,21-diol-20-one, pregnane-3α,17α,20ξ,21-tetrol and pregnane-3α,17α,21-triol-20-one in the urine (43). The neutral fraction of urine steroids of a man suffering from congenital adrenal hyperplasia also were separated with the aid of the Girard-T reagent and then into separate α and β hydroxysteroid fractions using digitonin, affording 22 previously described steroids of which the major "non-ketonic" steroids were pregnane-3α,17α,-20α-triol and 11-keto-pregnane-3α,17α,20α-triol as well as two new metabolites, allo-pregnane-3α,17α,20α-triol (XVIII, R = R″ = H, OH, R′ = R‴ = H_2) and pregnane-3α,11β,17α,20α-tetrol (64). The phenol estrogens were removed with alkali from female adrenal tumor urine extract and the 17-ketosteroids separated as above giving dehydroisoandrosterone, 3-chloro-Δ^5-androsterone, probably formed in the acid hydrolysis of the former, 3α-hydroxy-etiocholane-17-one, small amounts of androsterone (XXII), and a new 3α-hydroxyandrostene-17-one with the double bond in an undetermined position (205).

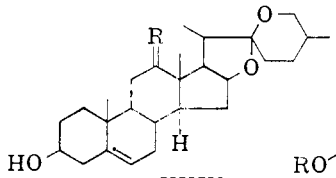


XXIII

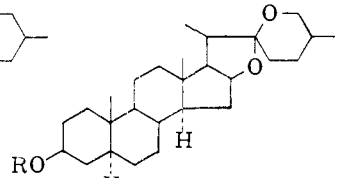
The urine steroids of a man treated with 21-desoxytetrahydrocortisone (3α,17α-dihydroxypregnane-11,20-dione) (XVIII, ring A/B *cis*, R = H, OH, R′ = R″ = O, R‴ = H_2) were similarly fractionated giving 3α,17α - dihydroxypregnane - 11,20 - one, androsterone (XXII), etiocholanolone and 11-ketoetiocholanolone in the ketonic fraction and 3α,17α,20α-trihydroxypregnane-11-one in the "nonketonic" fraction (146). These C_{19}-steroids have been isolated from the ketonic fraction of the urine of a pregnant cow administered progesterone (XXIII): $\Delta^{1,4}$-androstadiene-3,17-dione, androstane-3,17-dione, etiocholane-3,17-dione and Δ^4-androstene-3,17-dione (119).

The glucosiduronate residue from infant meconium was fractionated first with Girard-T reagent and then with digitonin and the Zimmerman chromogens determined on the glucosiduronate keto α and β fractions (62). Similarly the sodium pregnanediol glucuronidate fraction of human pregnancy urine was separated into nearly pure sodium pregnane-3α,20α-diol glucuronidate in the nonketonic fraction and what seemed to be sodium pregnane-3α-ol-20-one glucuronidate from the ketonic fraction (174).

Steroid keto-sapogenins also can be separated *via* their Girard-T hydrazones. Thus botogenin (XXIV, R = O) (gentrogenin, 20α,22a,25D-spirost-5-en-3β-ol-12-one) and neobotogenin (correllogenin, 20α,22a,-25L-spirost-5-en-3β-ol-12-one) were isolated from the crude sapogenin mixture from *Dioscorea spiculiflora* tuber (191, 192), and recently (74) neobotogenin was separated from the sapogenins of *Dioscorea chiapasensis*, the "nonketonic" fraction giving yamogenin (XXIV, R = H_2,C_{22} normal) and diosgenin (XXIV, R = H_2,C_{22} iso) as well as two new sapogenins chiapogenin and



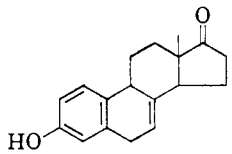
XXIV



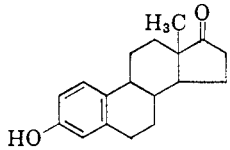
XXV

isochiapogenin shown to be 12β-hydroxyyamogenin and 12β-hydroxydiosgenin, respectively. Similarly tigogenin acetate (XXV, R = Ac) was separated from hecogenin acetate (12-ketotigogenin) and 23-keto-neotigogenin and 23-ketotigogenin separated in 8.8 and 2.5% yield, respectively, from the oxidation products of neotigogenin and tigogenin as their acetates, which contained neotigogenic acid in the first case as well as much unchanged starting material (32). Ethylene glycol was used as solvent since the usual technique led to the formation of emulsions.

The Girard separation method has been used in a number of cases to separate steroids labeled with carbon-14. Thus estradiol-17β-16-C^{14} (XX, R = H, OH) was administered to a woman suffering from breast cancer and the phenolic fraction of the urinal steroids separated into a ketonic fraction from which estrone (XX, R = O) and 2-methoxyestrone, a new metabolite, were isolated. The administration of estrone-16-C^{14} (XX, R = O) also gave a radioactive peak proceeding that of estrone in a countercurrent distribution and attributable to the methoxyestrone (99). In a study of steroid biosynthesis *in vitro* sodium acetate-1-C^{14} was administered to a pregnant mare and the urine and blood steroids processed. The neutral β-ketone fraction afforded 3β-hydroxyallopregnane-2-one and allopregnanolone comprising 40% of the β-ketones, as well as allopregnane-3β,20β-diol and allopregnane-3,20-dione. All these substances were radioactive. However when unlabeled pregnane-3α,20α-diol and pregnane-3,20-dione were added to the extracts and separated out they were devoid of radioactivity, showing that no 5α-steroids were synthesized in the animal body. The estrone isolated from the phenolic fraction had a radioactivity parallel to and of the same order as that of allopregnanolone over a period of days, suggesting that estrone and allopregnanolone and allied compounds are formed by similar pathways from acetate. The equilin (XXVI) and equilenin (XXVI, ring B aromatic) isolated had similar isotope concentrations but lower values than those of estrone suggesting that the ring-B



XXVI



XXVII

unsaturated estrogens are derived from acetate by a different mechanism (157). In this study the Girard-T hydrazones were hydrolyzed at pH 1.0 by adding concentrated hydrochloric acid and the ketones extracted with methylene chloride after standing overnight and heating to boiling.

Apart from their uses in the isolation of steroid ketones from mixtures, the Girard reagents have been used to purify such ketones contaminated with nonketonic material. Thus allopregnane-3-ol-one (XXI), pregnane-3-ol-20-one, and androsterone were prepared by hydrogenation of the corresponding diones and purified by Girard-T separation (113), and the acetate of epi-allopregnanolone was separated and purified in the same manner (111). In the preparation of ethynylandrostenediol the unchanged starting material, *trans*-dehydroandrosterone, was removed by Girard-T extraction (148). Similarly estrone (XX, R = O) was removed with Girard-T reagent from its irradiated product lumiestrone (XXVII), which does not react with this reagent (26, *cf.* 27). Unreduced ketone was removed in the same manner from the reduction products of 16-hydroxyestrone with Girard-T reagent giv-

ing 70% "estriol-like" and 30% "16-epiestriol-like" Kober chromogens (114).

Pregnenolone-20-semicarbazone on Oppenauer oxidation gave progesterone-20-monosemicarbazone which could not be recrystallized but on treatment with Girard-P reagent gave 44% pure progesterone (XXIII), the semicarbazone being hydrolyzed at the same time as the Girard hydrazone (71).

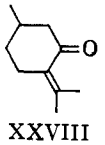
The formation of a Girard derivative has been used as a criterion of the presence of a ketone group in a compound. Thus the sodium excreting factor was shown to contain one keto-group and later identified as 3β,16β-diacetoxy-5α-pregnane-20-one (127). Similarly Reichstein's substance A (allopregnane-3β,11β,17α,20β,21-pentol) (XVIII, R = R′ = R″ = R‴ = H, OH) gives a monoketol (androstane-3β,11β-diol-17-one) on treatment with lead tetraacetate or periodic acid, whose acetylated derivative gives a Girard-T hydrazone (171).

The chromatography of steroid Girard hydrazones is dealt with in section VI, and relative rates and completeness of Girard hydrazone formation is covered in section VIII.

D. Application to Aldehydes

Aldehydes, both aromatic and aliphatic, react readily with the Girard reagents. Thus the Girard-T and -P hydrazones of hexaldehyde, citral, piperonal, vanillin, atranal, cinnamaldehyde, diphenylacrolein and *p*- and *m*-hydroxy-, *m*-nitro-, and 3,4-dihydroxy-benzaldehyde have been prepared (105), as well as the Girard-P derivatives of acetaldehyde, propionaldehyde, butyraldehyde, isovaleraldehyde, *n*-heptaldehyde, and crotonaldehyde (60). It has been reported that aldehydes even react rapidly in the absence of acetic acid reacting completely in hot ethanol (31). It was reported originally by Girard and Sandulesco (70) that Girard hydrazones of aldehydes are difficult to hydrolyze but later workers have shown that such derivatives are rapidly hydrolyzed by acid (31, 60, 105) (see section VII below).

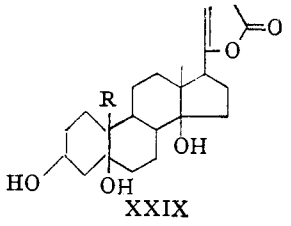
Aldehydes are often found in the "ketonic fraction" isolated in a normal Girard separation. Thus acetaldehyde was observed among the ketones isolated with Girard-T reagent from skim milk and shown to be responsible for its "cardboard" flavor (60), and decanol was separated in the same way along with carvone (XV) and pulegone (XXVIII) from orange oil (197).



XXVIII

Cardiac glycosides containing a formyl group also react rapidly and completely. Strophanthin glycosides were treated with a 50% excess of Girard-T reagent at 18° in the presence of acetic acid (158). The unchanged aldehyde-free compounds were extracted and the aldehydes recovered by shaking for 8 days with 1 *N* hydrochloric acid in the presence of chloroform. 2-Desoxy sugars are hydrolyzed by this treatment, cymarin (strophanthidin 3-cymaroside) giving only strophanthidin (XXIX, R = CHO). In some cases, such as convallatoxin (strophanthidin 3-rhamnoside) the hydrolysis is slow and the yield is improved by adding benzaldehyde.

The same procedure has been used to separate the glycosides of *Strophanthus ledienii*, which contained emicymarin (periplogenin digitaloside), strophanthidol (XXIX, R = CH_2OH), strophanthidin (XXIX, R = CHO), and periplogenin (XXIX, R = Me) as well as traces of an unidentified substance. These substances were further separated by paper chromatography after mild acid or enzyme hydrolysis (81).



XXIX

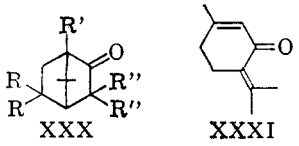
The separation of the various chlorophyll components is usually carried out by using their different behavior to buffers of various pH or from their different hydrochloric acid numbers, but the various carbonyl functions in these substances can also be used to effect a further separation (199). Thus the 3-formyl group in the *b*-series of compounds reacts readily with Girard-T reagent (5 minutes refluxing in acetic acid–ethanol), whereas the keto groups found in some of the *a*-compounds such as pheophorbide (β-ketoester) and purpurin 7*a* trimethyl ester (α-ketoester) react slowly if at all. *a*-Chlorin *p* trimethyl ester, *b*-chlorin *p* trimethyl ester, and purpurin 7*a* trimethyl ester and its 7*b* isomer were separated readily, although the ester groups were partially hydrolyzed and had to be reësterified with diazomethane before ether extraction. Pheophytin *a* and *b* could not be so separated, presumably since the phytol residue in the pheophytin *b* Girard derivative renders this compound ether soluble.

The decomposition on refluxing of aromatic aldehyde hydrazones to give azines (101, 102, 103) is dealt with in the following section.

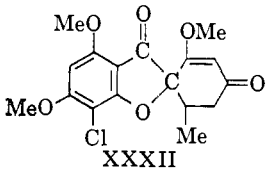
E. Anomalous Reactions

A number of carbonyl compounds have been found not to react with the Girard reagents. These are usually sterically hindered ketones such as dimethylcamphor (XXX, R = H, R′ = R″ = CH_3) (70), or α,β-unsaturated ketones which were generally unreacted. Dibenzylidene acetone does not react (131), although monobenzylidene acetone does (33). Piperitenone

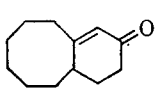
(XXXI) (*p*-mentha-1,4-diene-3-one) does not form a Girard hydrazone (126), but it does give a 2,4-dinitrophenylhydrazone (100). Griseofulvin (XXXII) reacts normally with Girard-P reagent and with 2,4-dinitrophenylhydrazine, but forms a pyrazoline with semicarbazide. It does not react with thiosemicarbazide under normal conditions, but gives a crystalline thiosemicarbazone on acidification of a solution of its Girard-P hydrazone (72). Of the two products XXXIII and XXXIV formed in the same reaction only compound XXXIII reacts with Girard-T reagent (36).

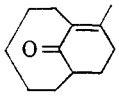

XXX

XXXI

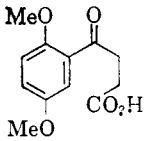

XXXII

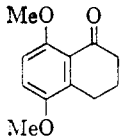
Both 4-(2,5-dimethoxyphenyl)-4-ketobutyric acid (XXXV) and 1,4-dimethoxy-α-tetralone (XXXVI) show no reaction toward Girard-T reagent, although the


XXXIII

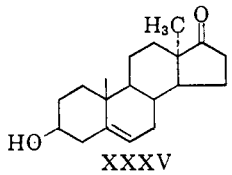

XXXIV

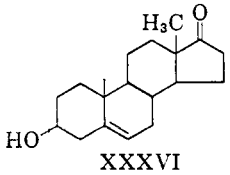
tetralone (XXXVI) forms a semicarbazone (36).


XXXV


XXXVI

Among steroid ketones the α,β-unsaturated ketone 16-benzylidene-*trans*-dehydroandrosterone and 21-benzylidene-5-pregnenolone do not form Girard-T derivatives (184), and doubly α,β-unsaturated ketone such as $\Delta^{1,4}$-3-keto-steroids (1,2-dehydrocortisone acetate and 1,2-dehydrocortisol and its acetate) do not react with either Girard-T or -P reagent and can accordingly be separated from Δ^1-3-ketosteroids, Δ^4-3-ketosteroids, $\Delta^{4,6}$-3-ketosteroids, and 3-ketosteroids which react (128). 11-Keto steroids are normally very unreactive and ketones such as $3\alpha,17\alpha,20\alpha$-trihydroxypregnane-11-one (64, 146) appear in the "nonketonic" fraction in the Girard separations. The ketones $3\alpha,17\alpha$-dihydroxypregnane-11,20-dione and 11-ketoetiocholanolone are found in the ketone fraction (146), presumably since they react at the 20 and 3 ketone groups, respectively. 3,21-Diacetoxy-17-hydroxypreg-5-ene-20-one reacts slowly with Girard-T reagent but not with semicarbazide (75). $3\beta,11\alpha$-Dihydroxy-12-ketocholanic acid forms a 2,4-dinitrophenylhydrazone but not a Girard-T hydrazone (19). $3\beta,17\alpha$-Diacetoxypregn-5-ene-20-one has been reported not to react with either Girard-T reagent or hydroxylamine acetate, but on treatment with potassium carbonate in methanol and Oppenauer oxidation gives 17-acetoxyprogesterone which reacts with Girard-T reagent (149), presumably at position-3. 17-Ketosteroids with ring C/D *cis* react with Girard-T reagent whereas their ring C/D *trans* isomers react normally. Thus lumiestrone (XXVII) (26, 27) and lumiandrosterone (XXXV) (18 iso compounds) (14) are unreactive while both estrone (XX, R = O) and androsterone (XXXVI) react readily.


XXXV

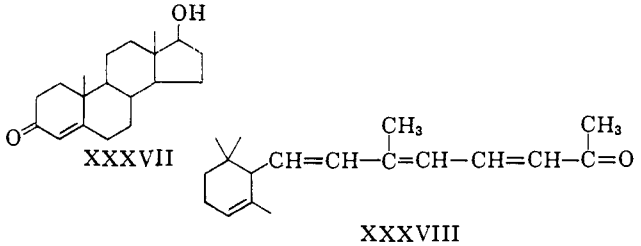

XXXVI

The sapogenins gofruside (corotoxigenin-D-allo-methyloside) (87) and strophanthidin (XXIX, R = CHO) (97) with an angular formyl group are reported not to form Girard derivatives, and plumieride acetate does not react with either Girard-T reagent or hydroxylamine acetate (159). Similarly pseudostrophanthidin which contains a masked aldehyde group in a 9,19-lactol bridge shows no ketone reactions (91, 92, *cf.* 44) and does not react with Girard reagents (81).

The normal Girard separation which depends on the fact that the Girard hydrazones are water soluble and that the nonketonic components are extractable with ether cannot be applied to the separation of ketonic substances from water soluble nonketonic substances such as sugars. Moreover if the ketones are themselves readily soluble in water the problem arises of extraction after liberation from their hydrazones. It has been proposed that the water-soluble ketones be recovered after extraction of the nonketonic compounds and acid hydrolysis, by adding equal parts of methanol and ether to the solution to precipitate the Girard-T reagent and then evaporating the solution after filtration to recover the ketones, which are free from Girard reagent and sufficiently pure to be used in biological assays (193). Occasionally the Girard hydrazones are insoluble or only slightly soluble in water. Thus the Girard-T and -P hydrazones of pregnane-5,16-diene-3-ol-20-one precipitate and can be recrystallized from water, although the corresponding derivatives of preg-5-ene-3-ol-20-one are soluble (188). Tritriacontan-17-one gives a Girard-P hydrazone which is only slightly soluble in water and the Girard-T derivative can only be dissolved in a large excess of ethanol (46). In this case the impurities could not be ether extracted but were removed by filtration. The fact that pheophytin *a* and *b* cannot be separated since the phytol residue in the *b* compounds renders it ether soluble (199) has been commented upon in section IVD.

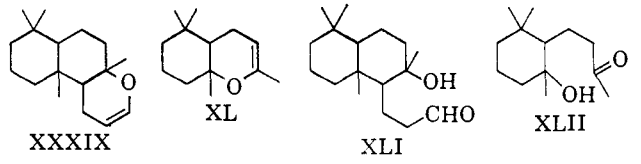
The rather vigorous conditions of the acid hydrolysis of the Girard hydrazones can cause dehydration in the

case of β-hydroxyketones. Thus 17-acetoxy-5-hydroxy-androstan-3-one gives a soluble Girard-T hydrazone which liberates testosterone (17-hydroxy-androst-5-en-3-one, XXXVII) on acid hydrolysis. The parent compound is not dehydrated by boiling with acetic acid in methanol but is dehydrated easily by alkalies (93). Although it has been suggested that dehydration occurs in the formation of the Girard hydrazone (36) it seems more probable that the dehydration and hydrolysis of the acetoxy group occurs during the hydrochloric acid hydrolysis of the hydrazone. Similarly ethyl 3-keto-5,19-dihydroxyetiocholanate affords ethyl 3-keto-19-hydroxy-Δ^4-etiocholenate (44), and ethyl 3-keto-5-hydroxy-8,9-oxidoetiocholanate is similarly dehydrated to ethyl 3-keto-8,19-oxido-Δ^4-etiocholenate (45). 3-Keto-15-hydroxy-14-iso-17-iso-21-norpregnane-19,20-dioic acid 20 ethyl ester gives ethyl 3-keto-14-iso-17-iso-19-nor-Δ^4-etiocholenate on acidification of its Girard hydrazone at room temperature (dehydration of the 5-hydroxy group and elimination of the 19-carboxy group) but gives an unidentified product at 100° (44). 6β,16α-Dihydroxy-3,5-cyclo-androstan-17-one under the acid conditions of a Girard-T separation was converted into 16α-hydroxydehydroepiandrosterone through loss of the 6β-hydroxy group and opening of the cyclopropane ring (61). The acid sensitive ketone XXXVIII, 8-(2,6,6-trimethylcyclohex-2-enyl)-octa-3,-



XXXVII XXXVIII

5,7-triene-2-one is rearranged to the fully conjugate ketone, but forms an unrearranged hydrazone with Girard-P reagent in the absence of acetic acid (96). The dihydropyranes XXXIX and XL give the Girard-T hydrazones of the corresponding hydroxy-



XXXIX XL XLI XLII

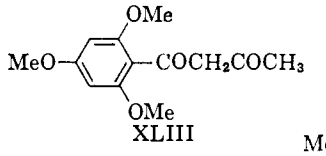
carbonyl compounds XLI and XLII, respectively (37, 173) and the compounds themselves on acid hydrolysis, although XLII is readily converted to XL on acidification (173). 2-Iodoadrenochrome and 2-iodo-epinochrome are reported (169) to give adrenochrome and epinochrome on treatment with Girard-T reagent, but 2-bromoadrenochrome gave no isolatable product.

Since acetic acid is usually used as catalyst in the formation of the Girard hydrazones hydroxy groups present in such compounds as steroids may be acetylated under these conditions and it has been advised that the ketonic products be either treated with potassium bicarbonate in aqueous methanol (21) or that the ketones be regenerated by heating with hydrochloric acid (107) to hydrolyze any acetate formed. It was observed that the 7β-hydroxycholesterol submitted to a Girard-T separation in methanol was converted into its 7-methyl ether (76). Other workers had carried out similar separations of this compound in methanol (137, 138) and ethanol (12, 13) without recognizing that etherification had occurred. 3-Acetoxy groups are stable to Girard separations but 21-acetoxy groups are partially hydrolyzed (73).

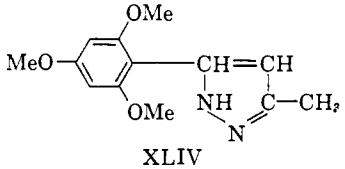
Both pregnane-3α,20α-diol glucuronic acid and pregnane-3-ol-20-one glucuronic acid are stable under the conditions of forming their Girard-T derivatives but in the ether extraction of the nonketonic fraction the customary neutralization of $^9/_{10}$ of the acetic acid is omitted since the glucuronic acids are otherwise highly ionized and are not extracted by ether. To avoid hydrolysis of the hydrazones and achieve a good separation the extraction should be carried out rapidly at a low temperature (174).

A number of aromatic aldehydes were found to give between 3 and 30% aldazines on heating with Girard-T or -P reagent in acetic acid in methanol (101). This was first attributed to the presence of free hydrazine in the Girard hydrazides but pure Girard-P reagent on reaction with salicylaldehyde still gave 0.5% azine and recovery of 92% of the aldehyde on hydrolysis (101, 103). However later workers (35) showed that the azine is formed indirectly from hydrazine liberated by the solvolysis or alcoholysis of excess Girard reagent. These workers noted that estrone azine often is isolated in the Girard separation of estrone from hormone extracts, and the azine being insoluble in water appears in the ether "nonketonic" extract. Estrone Girard-P hydrazone gave 6% estrone azine after 2 hours and 36% azine after 24 hours. Refluxing Girard-P hydrazide in acetic and ethanol for 24 hr. and then adding estrone and refluxing for 2 hr. more gave a 36% yield of estrone azine. If the estrone Girard-P hydrazone had itself decomposed to give estrone azine, then only 6% of the azine would be expected to be formed. It was suggested that hydrazine liberated by the hydrolysis of alcoholysis of the excess Girard-P reagent, or of this reagent itself liberated by hydrolysis of the once-formed Girard-P hydrazone reacts to give the normal hydrazone which decomposes to the azine in the presence of acid (175). Dehydroiso-androsterone, Δ^5-pregnenolone and testosterone all give from 5 to 10% azine with Girard-P reagent. However the Girard-T reagent gives much less azine, estrone and Girard-T hydrazide giving only 10% azine in 24 hr. and none in 2 hr. It is accordingly preferable to use the Girard-T reagent rather than the

Girard-P reagent particularly if prolonged heating must be used to form the hydrazone. The aldazine of β,β-diphenylacrolein was also isolated during the separation of ketones formed in the oxidation of 24,24-diphenyl-chola-4,20,23-triene-3-one with Girard-P reagent and was considered to be formed by the hydrolysis of the reagent (118). An insoluble product formed in the preparation of the Girard-P derivative of 2,3-dihydro-3-keto-1H-pyrido[3,2,1-kl]phenothiazine (X) (109), later was shown to be its azine (110), also being formed directly from the ketone itself and hydrazine sulfate. The compound also was formed by treating the pure Girard-P hydrazone of X with an equimolar amount of the same ketone, and was resistant to concentrated hydrochloric acid or concentrated potassium hydroxide, being hydrolyzed to the ketone only with concentrated hydrochloric acid and dioxane. Girard-T reagent also gives this same azine but less readily. It has been reported also that the β-diketone eugenone (XLIII) gives the pyrazolone XLIV on treatment with Girard-P



XLIII



XLIV

reagent (160), presumably being formed by the reaction of the diketone with hydrazine liberated from the hydrolysis of the reagent.

V. Separation of Mixtures of Ketones

A. General Applications

The observations in qualitative experiments that some ketones react more rapidly with the Girard reagents than others has only been applied in a few cases to the separation of two or more ketones by selective reaction with the more reactive ones. Piperitone (XXXI) does not react with Girard-T reagent (100) and can be separated from isomenthone (XII), the corresponding saturated ketone in geranium oil (5), since the latter reacts readily. Similarly of the two ketones XXXIII and XXXIV only the former reacts with Girard-T reagent and can be separated in this manner (36). The separation of *a*-keto and *b* chlorophylls, due to the rapid reaction of the formyl group in the *b* chlorophylls (199) has been mentioned already (section IVD).

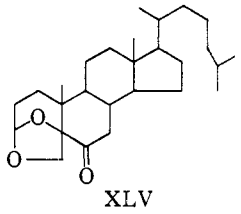
B. Steroids

The selective reaction of carbonyl groups in various positions in the steroid nucleus has found more application in the separation of steroid ketones. The fact that 11-ketosteroids do not react with Girard-T reagent and are found in the "nonketonic" fraction (64, 146) is mentioned above (section IVC).

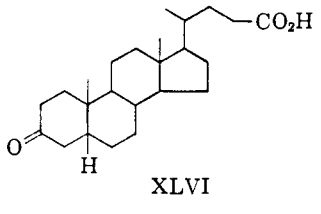
Ketone 104, an oxidation product of cholesterol, can be separated from cholestenone since it reacts much less readily with Girard-T reagent, while the cholestenone reacts completely in 1 hour at room temperature (in methanol–acetic acid) and can be recovered in 91% yield (54, 55, 58). Ketone-104 was later shown to be 3,4-secocholestane-6-one-(3α,5α) (3β,4)-dioxide (XLV) (57) and its slow rate of reaction must be a result of the fact that the ketone is hindered.

The large difference in the rate of reaction of 3- and 17-keto-steroids (see section VIIIB) was used in separating a mixture of cholestan-3-one and estrone (XX, R = O) as its methoxy ether, by reaction with only one equivalent of Girard-T reagent (202), and pregnane-3,-20-dione reacted successively with one equivalent of Girard-T reagent to form the 3-monohydrazone, with one equivalent of sodium borohydride in alkaline solution to reduce the 20-keto group and the 3-hydrazone then hydrolyzed with acid to give pregnane-20α-ol-3-one as the principal product (202).

The relatively slow reaction rate of α,β-unsaturated ketones has been used to separate them from the more reactive saturated ketones. Thus 21-acetoxy-17α-hydroxypregna-9-ene-3,20-dione was separated from 21-acetoxy-17α-hydroxypregna-4,9-diene-3,20-dione by treating the former with Girard-T reagent and recovering it by hydrolyzing the aqueous extracts (53). The Girard-P reagent also has been used to separate 21-acetoxy-3β,17α-dihydroxy-5α-pregna-9(11)-ene-20-one from 3β,17α-dihydroxy-5α-pregna-9(11)-ene-20-one (50).



XLV



XLVI

Since the Girard-T steroid hydrazones are salts readily soluble in water and buffer solutions they provide ideal derivatives for use in electrophoresis (108). The Girard hydrazones prepared in 10% acetic acid in methanol can be separated readily in 0.05 *M* sodium borate, the hydrazones being detected by spraying with Kraut–Dragondorff reagent or by viewing under a quartz mercury lamp. Good separations of progesterone, testosterone, and estrone are achieved, the hydrazone of progesterone moving ahead of the monohydrazones. The relative mobilities (to progesterone 1.00) were androsterone 0.85, desoxycorticosterone 0.83, methyltestosterone 0.77, ethyltestosterone 0.76, testosterone 0.75, and estrone 0.53, all in sodium borate solution. This electrolyte proved to be the most suitable since

the monohydrazones of testosterone and estrone move at the same rate in acetate buffer (pH 4.5) or diethylbarbiturate buffer (pH 8.6). Moreover, cortisone acetate moves just behind testosterone in diethyl barbiturate buffer (in 20% methanol) and progesterone and testosterone also have similar mobilities. Hydrocortisone gave two spots, probably from the mono- and dihydrazones. Electrophoretic separation has been suggested for the analysis of steroid hormones in pharmaceutical preparations and is more convenient than a previously described method of esterification of the Girard-T derivatives before use in the electrophoresis (190), which had been applied to the separation of the ketosteroids in the neutral ketonic fraction obtained from urine.

Girard-T hydrazones also can be separated by paper chromatography (104). The positions of the hydrazones are revealed by spraying with iodoplatinate solution (see below), whereby a few micrograms of ketosteroids can be detected (25). α-Ketocorticoids, however, are sufficiently polar to be used as such in chromatography with polar solvents (211). In butanol:propanol:water (6:2:1) increasing the number of keto-groups in a steroid lowers the R_f value of its Girard-T hydrazone (176). 3-Keto groups in methyl 3-ketocholanate (XLVI), 3-keto-12-hydroxy-cholanate, 3-keto-7,12-dihydroxycholanate, 3-keto-12-acetoxycholanate, and 3-keto-7,12-diacetoxycholanate did not react. The R_f values obtained for some 7- and 12-ketosteroids were 12-ketocholanate 0.83, 12-keto-3-acetoxycholanate 0.76, 12-keto-3-hydroxycholanate 0.74, 3,12-diketocholanate 0.70, 7-keto-3,12-dihydroxycholanate 0.58, 12-hydroxy-3,7-diketocholanate 0.53, 7,12-diketocholanate 0.42, 3-hydroxy-7,12-diketocholanate 0.23, and 3,7,12-triketocholanate 0.09 (all as their methyl esters).

Aromatic, heterocyclic, and saturated aliphatic aldehydes can be separated as their Girard-T hydrazones using 1-butanol:ethanol:water (27:3:10) as eluent and revealed by spraying with iodoplatinate solution. The compound can then be recovered from the spots by elution with methanol (162). The Girard-T hydrazone of adrenochrome is best eluted with pyridine–water (6.5:3.5) or with aqueous buffer of pH 7.4 in which it is stable, whereas adrenochrome itself is best eluted with ethanol–water (59).

VI. Formation of Solid Complexes

Girard and Sandulesco in their original publication (70) reported that the Girard hydrazones did not form picrates in conveniently dilute solution, but gave insoluble complexes with potassium bismuth iodide solution as an immediate orange flocculent precipitate which slowly forms a red crystalline precipitate. The precipitate is soluble in ether and hence ether should be eliminated first from the solution. The Girard hydrazones also form precipitates with mercuric iodide in potassium iodide solution and this is a very sensitive test for these compounds since 30 γ per ml. of cinnamaldehyde can be detected. This method has been developed for the analysis of steroid ketones (84), by filtering off, washing, and weighing the precipitates. Dehydrodesoxycholic acid, dehydrochloric acid, cholestanone, estrone, and progesterone gave carbonyl values of 96.9–101.7%. In the analysis of dehydrodesoxycholic acid in bacterial media 96.6–99.7% of that added was recovered (84). Similarly 3-hydroxy-12-ketocholanic acid, 3,12-diketocholanic acid, 3-hydroxy-7,12-diketocholanic acid, and 3,7,12-triketocholanic acid added to bile were recovered in 97.1 to 101.2% yield (85).

Potassium iodoplatinate has been shown to give precipitates with steroid Girard hydrazones which can be used to reveal the spots of these compounds in paper chromatography (see above) with which they give purple spots with amounts as small as 20 γ. Potassium iodobismuthate similarly gives orange spots (210).

VII. Hydrolysis of Derivatives

A. General Conditions

Girard and Sandulesco (70) originally reported that aldehydes formed Girard hydrazones which were very resistant to hydrolysis and did not recommend their reagents for use with aldehydes. However later workers made use of both Girard-T and -P reagents to separate aldehydes and hydrolyzed the hydrazones with either hydrochloric acid or sulfuric acid at room temperature for 12 to 24 hr (101, 105). These conditions seem to be excessive since it has been reported that the Girard-P hydrazones of simple aldehydes such as acetaldehyde, propionaldehyde, butyraldehyde, isovaleraldehyde, *n*-heptaldehyde, and crotonaldehyde are hydrolyzed rapidly at room temperature in 2 *N* sulfuric acid, as are the derivatives of simple ketones such as acetone, methyl ethyl ketone, amyl methyl ketone, and mesityl oxide (60). Yields of 80–90% were obtained in the hydrolysis at room temperature of the Girard-P hydrazones of heptaldehyde, *m*- and *p*-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, piperonal, and citral. However, it has been reported recently that citral could not be recovered from its Girard-T hydrazone by acid hydrolysis but was regenerated by standing at room temperature with a large excess of formaldehyde (180). *n*-Octanal, 2-octanone, acetophenone, and benzaldehyde as well as citral were thereby recovered in 72–96% yield. Girard and Sandulesco (70) recommended the use of formaldehyde in the presence of acid.

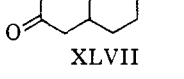
For compounds sensitive to acid, such as glycosides, hydrolysis of the Girard-T hydrazone has been effected by shaking with 1 *N* hydrochloric acid for 8 days in the presence of methylene chloride (158), although 2-desoxy sugars are hydrolyzed by this procedure. In the hy-

drolysis of water-soluble ketonic compounds the liberated Girard hydrazine can be precipitated by adding methanol (2–3 volumes) and ether (15–20 volumes), filtering off the Girard reagent and then evaporating the filtrate to recover the ketonic material (193).

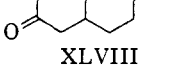
B. Selective Hydrolysis

It was first reported by Girard and Sandulesco (70) that the Girard hydrazones of α,β-unsaturated ketones, such as cholestenone and testosterone, were resistant to acid hydrolysis, although the ketones themselves react very rapidly. This difference between the behavior of saturated and unsaturated ketones was put to good use by Reichstein in his fractionation of the adrenal corticoid steroids (141) when he selectively hydrolyzed the Girard-T hydrazones by increasing the acid concentration thereby separating first the saturated (the allopregnane group) and then the unsaturated (the cortisone group) ketonic compounds.

Fractional hydrolysis has also been used to separate α-ionone from dihydro-α-ionone (151), since the Girard-P hydrazone of the former ketone is not hydrolyzed at pH 5, at which pH the hydrazone of the latter is easily split. Similarly *cis*-dihydro-ionone was separated from a mixture of ketones (6) by fractional hydrolysis of the Girard-P hydrazones at increasing pH. Recently *trans* - 2 - keto - 6 - methoxy - 12 - methyl - 1,2,3,4,-9,10,11,12-octahydrophenanthrene (XLVII) was sepa-



XLVII



XLVIII

rated from the related α,β-unsaturated ketone, *trans*-2-keto-6-methoxy-12-methyl-1,2,9,10,11,12-hexahydrophenanthrene (XLVIII) using the fact that the Girard-T hydrazone of the former (XLVII) was hydrolyzed at pH 5–6, whereas the corresponding derivative of the latter (XLVIII) was only hydrolyzed at pH < 4 (99a).

VIII. QUANTITATIVE ASPECTS OF THE REACTION

A. Determination of the Reagents

It has been shown recently that the Girard-T reagent can be titrated iodometrically under certain conditions (201). The reaction with iodine is slow in acid solution, but proceeds rapidly at pH 7–8, being complete and corresponding to the equation analogous to the determination of semicarbazide (10)

$$(CH_3)_3N^+CH_2CONHNH_2 + 2I_2 + H_2O \rightarrow (CH_3)_3N^+CH_2CO_2H + N_2 + 4HI$$

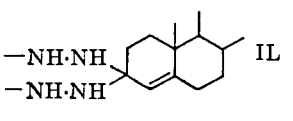
Above pH 8 the iodine begins to react with the base and the best conditions are using a phosphate buffer of pH 7. The Girard-P reagent, however, only reacted slowly with iodine in both acid and alkaline solution even on warming.

The ketone content of essential oils has been determined by treating with an excess of Girard-T reagent in aqueous ethanol and then titrating the excess (203). The results are similar to those given by oximation (63, 153) since only saturated ketones react rapidly.

B. Kinetics of Formation of Hydrazones

Girard and Sandulesco in their original publication (70) report from qualitative observations that the order of rate of formation of Girard hydrazones of ketones is methylalkyl > alicyclic > methylaryl > diaryl. They also report that acetic acid greatly accelerates the reaction, cyclohexanone reacting only in several hours in methanol but in a few minutes in methanol containing acetic acid. In a recent study (102) it was found that the rate of reaction of cholest-4-ene-3-one in methanol with Girard-T reagent was very slow, being greatly accelerated by acetic acid. However, at high concentrations (2.5 *M*) the reaction was not complete, since the reverse reaction of hydrolysis is catalyzed by low pH or high acid concentration (see below). At 40° the rate of reaction (in 0.2 *M* acetic acid) was nearly twice that at 25°, corresponding to an energy of activation of 6.5 kcal./mole, but the reaction was not complete, indicating that the over-all reaction was exothermic. The rates of reaction of ergost-8(14)-ene-3-one and estrone methyl ether also increased with increasing acetic acid concentration, but the amount of reaction decreased, the reaction with estrone methyl ether even being incomplete in 0.2 *M* acid. The rates of reaction of a number of steroid ketones in 0.2 *M* acetic acid in methanol at 25° established the order of keto group reactivity of $3 > 6 > \Delta^4\text{-}3 \sim 7 \sim 17 > 20 > 12 >> 11$ and only in the cases of 3- and Δ^4-3-ketones were the reactions complete. The results showed an over-all difference of 4000, with 3- and 17-ketones and 3- and 20-ketones showing differences of 30 and 60 times, respectively. These large differences can be utilized to carry out selective reactions on these groups (see above).

The rates of reaction in 2-propanol at 25° and pH 3.5 were generally much faster, the order of reactivity being similar ($3 > 17 > 20 > 6 \sim \Delta^4\text{-}3$), but the differences were much smaller. However in all cases the steroid ketones reacted with more than one equivalent of Girard-T reagent to a maximum of two equivalents in the case of cholest-4-ene-3-one. The resulting solution had no select absorption in the region 220–240 mμ, and the original ketone and Girard reagent were regenerated on acidification suggesting that the product formed had structure IL.



IL

Some data are available concerning the completeness or effectiveness of Girard separations. In an experiment in which 1 g. of carrier cholesterol was added to 1.12 mg. of cholestenone-4-C^{14} the nonketonic fraction afforded nonradioactive cholesterol, indicating that the separation was complete. However in the separation of cholestenone from coprostanol the ketonic fraction always was contaminated wih the latter (145). Similarly in the separation of metabolites of 16-keto-estradiol-17β-16C^{14} the ketonic fraction on retreatment with Girard-T reagent gave a small nonketonic fraction which had the major radioactivity (106). In the separation of products formed in the incubation of desoxycorticosterone the nonketonic fraction gave a positive test with tetrazolium blue and had to be treated a second time so that it gave no reaction (177).

A preliminary report has been published of a detailed study of the completeness of the Girard-T separation of steroid ketones (73). Pure steroids were subject to a micro Girard-T separation and the ketonic and nonketonic fractions analyzed. Only 17-ketosteroids were completely recovered. Progressive introduction of hydroxyl or keto groups lead to decreased recovery in the ketonic fraction and the decreases were greatest for $\Delta^{1,2}$- and $\Delta^{16,17}$-unsaturation.

C. Kinetics of Hydrolysis of Hydrazones

The fact that Girard-T hydrazones are hydrolyzed at different rates was nearly used by Reichstein and others to separate saturated and unsaturated steroid ketones (see section VIIB) and the fact that cyclohexanone and other 6-membered ketone hydrazones are less stable in acid solution than cyclopentanone or 5-membered ring ketones is also reported (135, 206).

In a kinetic study (202) a number of steroid ketones reacted with an excess of Girard-T reagent at pH 3.5 for sufficient time (24 hr.) to form their hydrazones completely and then were hydrolyzed by adjusting the pH to 1.5. The rates of hydrolysis were determined iodometrically. The rates found for 3-, Δ^4-3-, 17-, and 20-ketosteroids were very little different, although at pH 3.5 17- and 20-ketosteroids are hydrolyzed more readily.

D. Spectra of Hydrazones

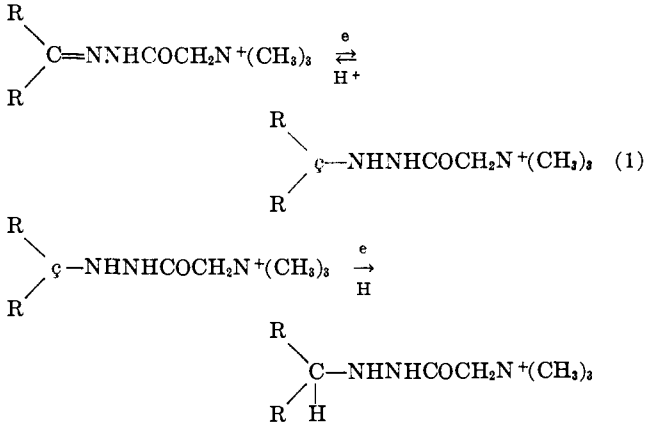
α,γ-Unsaturated ketones show an absorption maximum in the ultraviolet at 230–250 mμ (65) whereas their semicarbazones have maxima at 260–280 mμ. The solutions of Girard-T hydrazones of such ketones have a maxima at 280 mμ (82). Thus the Girard-T hydrazone of 17-hydroxy-11-dehydrocorticosterone (Kendall's substance E, XIX, R = R′ = H, OH) has a maximum at 281 mμ in a phosphate buffer of pH 7.0. The addition of hydrochloric acid regenerates the ketone and the maximum is displaced to 245 mμ. Similarly the hydrazones of desoxycorticosterone and testosterone have maxima at 281 and 283 mμ, respectively. The hydrazone of the saturated ketone dehydroisoandrosterone has its maximum at 239 mμ (*cf.* saturated ketones λ_{max} 230 mμ) (41). Beer's law is obeyed by these compounds in the presence of excess Girard-T hydrazine. The method was used to detect Kendall's compound E isolated from the urine of a patient treated with ACTH and it was suggested that α,β-unsaturated steroid ketones could be analyzed by measuring the intensity at 281 mμ of the Girard-T hydrazone and then of the ketone itself after acid hydrolysis. Cholest-4-ene-3-one on reaction with an excess of Girard-T reagent showed maxima at 280 mμ, ϵ 15,700 (as calculated from the amount of reacted reagent) (202).

Aliphatic ketones such as ethyl isobutyl ketone, *n*-propyl *t*-butyl ketone, methyl *t*-butyl ketone, and methyl isobutyl ketone gave Girard-T hydrazones with maxima at 230–240 mμ (209), but *t*-butyl isopropyl ketone gave no maximum, indicating that it does not react with Girard reagent. The ultraviolet spectra of the Girard-T and -P hydrazones of adrenochrome and of the Girard-T hydrazones of epinochrome and 2-carbethoxyepinochrome have been reported (169).

IX. POLAROGRAPHY OF GIRARD HYDRAZONES

A. Acyclic and Cyclic Ketones

Saturated ketones are only reduced polarographically at very negative voltages (*ca.* −2.2 v.) (98), although carbonyl derivatives such as hydrazones and phenylhydrazones are reduced more easily. In a detailed study of the polarographic reduction of Girard-T hydrazones of saturated acyclic and cyclic ketones (135) it was found that the wave height corresponded to a 2-electron reduction, while analysis of the wave points indicated a 1-electron potential determining step (step 1). The final product is a hydrazine, but the dimer of the product of step 1 could be isolated.



The wave height for the reduction of cyclohexanone Girard-T hydrazone was nearly constant in alkaline solution, but decreased considerably in acid solution.

The height, however, was increased by adding an excess of Girard reagent, indicating that a reversible hydrolysis takes place in acid media. The decrease in acid solution was greater with time, although the rate of decrease was much less in nearly neutral solution. The half-wave reduction potential decreased linearly with pH of the solution. The reduction at pH 8.2 was diffusion controlled, whereas it was rate controlled at pH 12.2.

Acyclic ketones, such as acetone, ethyl methyl ketone, diethyl ketone, methyl *n*-undecyl ketone, and di-*n*-octyl ketone had a constant value (−1.56 v. against the normal calomel electrode) for the reduction potential of their Girard-T hydrazones measured at pH 8.2, using 0.5% gelatin as maximum suppressor. However the half-wave reduction potentials of cyclic ketone Girard hydrazones measured under the same conditions, varied with ring size, being a minimum (−1.51 v.) for cyclohexanone, a maximum (−1.64 v.) for cycloöctanone, and nearly constant (−1.55 v., all against the normal calomel electrode) for ring ketones above 12 carbons. These values are consistent with the progressive development of folded ring configurations which confer greater stability on medium ring size compounds (*ca.* C_9). The dissociation constants of the cyanohydrins of these ketones showed similar trends (136).

The polarographic reduction of the Girard-T hydrazones of a number of dialkyl ketones showed a similar relation to their cyanohydrin dissociation constants (52. 209). *n*-Alkyl methyl ketone hydrazones gave half-wave potentials of −1.50 to −1.51 v. and branched chain compounds gave values 0.03–0.04 v. more negative (209). Direct comparison with the cyanohydrin dissociation constants (52) showed that the steric effect of the alkyl groups is more important than their inductive effect in polarographic reductions. Highly branched alkyl ketones, such as *t*-butyl isopropyl ketone, gave very low reduction wave heights (209) suggesting that only a very small amount of hydrazone is formed and the same has been found for bridge-ring ketones such as camphor (XXX, R = R″ = H, R′ = Me), isofenchone (XXX, R′ = R″ = H, R = Me) and camphenilone (XXX; R = R′ = R″ = H) (200).

B. Steroid Ketones

The polarographic reduction of Girard-T hydrazones offers a simple method for the determination of small quantities of 17-ketosteroids, 3-ketosteroids, 3-keto-Δ^4-unsaturated steroids, and 3-hydroxy-Δ^5-unsaturated steroids (98, 120). 17-Ketosteroids are not themselves reducible in ammonium chloride solution (206), but their Girard-T hydrazones are reduced at about −1.45 v., the reagent itself not being reduced until about −1.65 v. Androsterone, isoandrosterone, and dehydroisoandrosterone gave diffusion currents which were proportional to the concentration at low concentrations. A linear relation existed between the diffusion current and the concentration of hormone (0.05 to 1.0 mg. in 2.50 ml.) in the presence of traces of urine extracts, which appeared to stabilize the Girard hydrazones. Concentrations of 1 to 20 mg. of these hormones and estrone per liter of urine could be analyzed. Interfering ketonic material which occasionally gives a small reduction wave at −1.0 to −1.3 v. in some neutral urine extracts can be removed by oxidation with aqueous potassium permanganate in aqueous dioxane (7). The differentiation of these two waves is also improved by decreasing the rate of increase of the applied voltage (198).

A number of other similar reagents have been investigated for the polarographic reduction of 17-ketosteroids (9). Most gave much lower diffusion currents, only glycine hydrazide, maleic acid hydrazide, and aminoguanidine giving wave heights of the same order as the Girard derivatives and only the last two proving suitable for analysis.

The polarographic analysis gives lower results than the colorimetric method and shows a smaller variation due to errors in technique (8). It has been applied to analyze estrone in pregnant mare urine (16) and to analyze 3α- and 3β-hydroxy-17-ketosteroids after digitonin separation (28).

3-Ketosteroid Girard-T hydrazones are not sufficiently stable in acid solution to be analyzed polarographically (135, 206). However cholestanone can be thus analyzed in alkaline solution (135).

3-Keto-Δ^4-unsaturated steroids can be analyzed polarographically as such (49) giving half-wave reduction potentials of −1.5 to −1.6 v. in 0.75 *N* tetraethylammonium hydroxide in aqueous 2-propanol (135). However the Girard-T hydrazones of these ketones are reduced at a much lower voltage (*ca.* −1.23 v.) and can be distinguished readily from the wave of the 17-ketosteroids (at *ca.* −1.44 v.). Moreover the wave heights are much higher for the Girard derivatives than for the ketones themselves, being 6.3 times as high as for testosterone (206). Δ^4-3,20-Diketosteroids, such as cortisone, corticosterone, and desoxycorticosterone gave two reduction waves as their Girard-T hydrazones, one at −1.23 v. of the Δ^4-3-keto group and another at −1.45 v. of the 20-ketone.

Mixtures of androsterone with testosterone and with desoxycorticosterone were analyzed in this manner (206), as were methyltestosterone and preg-4-ene-17-ol-3-one in pharmaceutical preparations (156). Progesterone has been determined in blood to the lower limit of 0.1 μg./ml. in a 20 ml. sample (29, 30) and adrenocorticol hormones in human peripheral blood using a special micro-cell with recovery of 80% at the 5 to 10 μg. level (121, 122).

The polarographic estimation of Δ^4-3-ketosteroids has been extended to the determination of Δ^5-3-hydroxysteroids, which are first oxidized to the corresponding Δ^4-3-ketosteroids by the Oppenauer method with acetone in benzene in the presence of aluminum *t*-butoxide, and then were determined as above (79). Thus dehydroisoandrosterone gave two waves on polarographic reduction of the Girard-T hydrazones after Oppenauer oxidation, one at -1.2 to -1.3 v. corresponding to the Δ^4-3-keto grouping formed in the oxidation and another at -1.5 v. characteristic of the original 17-keto group, and also given by the unoxidized compound. Mixtures of androsterone and dehydroisoandrosterone can be analyzed by first determining the total 17-keto content and then oxidizing to determine the amount of Δ^4-3-keto compound formed in the oxidation of the latter. Cholesterol, stigmasterol, and phytosterol mixtures can be determined similarly after oxidation to the Δ^4-3-ketones (80). The neutral 17-keto steroids from the urine of a girl suffering from a corticoadrenal tumor were analyzed in this manner for dehydroandrosterone (205).

Derivative polarography, graphing the rate of change of current (di/dE) against the change of voltage (dE), has been applied to the analysis of C_{27} ketosteroids as their Girard-T hydrazones (144). 17-Ketosteroids gave half-wave reduction potentials of -1.68 v., Δ^4-3-ketosteroids -1.37 v. and cholesta-3,5-diene-7-one and cholesta-4,6-diene-3-one -1.24 and -1.18 v., respectively. Δ^4-3-Ketosteroids such as cholest-4,3-one, testosterone (XXXVII), progesterone (XXIII), corticosterone (XIX, R = H, OH, R′ = H, R″ = O) and 11-dehydrocorticosterone generally gave two waves whereas coprostan-3-one gave only one wave ($E_{1/2}$ -1.34 v.). The half-wave reduction potential of cortisone (XIX, R = R″ = O, R′ = OH) increased with pH but the derivative diffusion current peak decreased and then increased with pH. The height of the diffusion current peak was linear with concentration for 10^{-3} to 10^{-4} *M* solution.

X. Allied Reagents

Of the numerous reagents used to form soluble derivatives of carbonyl compounds (see section I) the most closely related to the Girard reagents (172) is dimethylaminoacethydrazide (L) known as reagent-D (9, 189). The compound is not hygroscopic and the hydrazones are hydrolyzed easily with cold acid, including the derivatives of aldehydes such as *m*-nitrobenzaldehyde and α,β-unsaturated ketones such as cholestenone (189). This reagent has been used (173) to isolate the hydroxy ketone derivative XLII of ionone.

$(CH_3)_2NCH_2CONHNH_2$ L

Another closely related compound is the N-methyl-β-carbohydrazide pyridinium *p*-toluenesulfonate VI. This gives derivatives of well-defined and usually high melting point which are soluble in water but almost insoluble in nonhydroxylic organic solvents. The derivatives are hydrolyzed easily by acid and have been used to identify the *n*-aldehydes of C_2 to C_{14} (1). Nicotinic hydrazide (reagent-N) (185, 187), lutidinedicarboxylic acid hydrazide (187) and benzylic hydrazide (186) give insoluble hydrazones with carbonyl compounds, and nicotinic hydrazide has been used to precipitate androst-4-ene-3,17-dione (185). A number of betaine derivatives have been used for the separation of physiologically active glycosides, the recovery of pregnanediol from pregnancy urine extracts, and of testosterone formed in the biochemical reduction of androstenedione. They react in the presence of pyridine and the products are precipitated as their picrates or chloroplatinates (150).

p-Hydrazinobenzene sulfonic acid (II) was early employed (15) in preparing derivatives of aldehydes and reacts readily in aqueous solution. The addition compounds formed with benzaldehyde, salicylaldehyde, anisaldehyde, and *m*-nitrobenzaldehyde are not true hydrazones, but could be recrystallized from boiling water. However the compounds formed with aliphatic aldehydes such as valeraldehyde as well as those formed with cinnamaldehyde and cuminaldehyde are hydrolyzed by boiling water (15, 181). Ketones form normal hydrazones and these are hydrolyzed readily with mineral acid and can be precipitated as their lead salts (181). This reagent as its sodium salt, which is soluble in ethanol, has been used to extract jasmone from jasmin oil, the ketones from castoreum and Algerian cypress oil (152), and menthone from peppermint oil (181).

The related *p*-carboxyphenylhydrazine (VII) was first used for the isolation of pyruvic acid formed in the alkaline decomposition of cysteine in aqueous solution from which the hydrazone was precipitated on acidification (34). It was later shown (2) that the hydrazones of the reagent could be split with either formaldehyde or pyruvic acid. The hydrazones of cholestanone and coprostanone are decomposed by aqueous formaldehyde whereas the hydrazone of cholest-4-ene-3-one is only hydrolyzed with pyruvic acid in boiling ethanol, and this difference can be used to separate these compounds. The hydrazones are easily oxidized and are usually prepared in an atmosphere of carbon dioxide. Derivatives of 65 different carbonyl compounds have been prepared (182). This hydrazide has been used to separate long-chain ketones from waxes (51). Thus *n*-nonacosane-12-one (myristone) and *n*-tetratriacontane-15-one-ol (coccerylic alcohol) were isolated from natural wax, *n*-nonacosane-15-one was extracted from cabbage-leaf wax and 10-hydroxypalmitone was separated from santal-leaf wax. These ketones react rather slowly and the addition of pyridine

accelerates the reaction (51). Aliphatic aldehydes of C_{16}-C_{18} were isolated in small quantities from tissues (4) using this reagent, as was 3-hydroxy-6-keto-allocholanic acid from hog bile (3).

Carboxymethoxylamine (aminoxyacetic acid, IX) also gives derivatives which contain a carboxylic acid group and are therefore soluble in alkali (2, 18). On acidification the oxime derivatives are precipitated and can be filtered off or ether extracted. Cholestanone was condensed with IX in the presence of sodium acetate and the derivative extracted with 1% aqueous potassium carbonate. Acidification of the carbonate solution liberated the oxime which was hydrolyzed with dilute hydrochloric acid in ethanol (2). The phenolic fraction from pregnancy urine was fractionated in this way to give estrone and α-estradiol (83, 204).

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