review

Higher aliphatic 2,4-diketones: a ubiquitous lipid class with chelating properties, in search of a physiological function

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Abstract A group of homologous 2,4-diketones with chain lengths from C_{13} to C_{25}, derived from fatty acids, is present in human and mammalian tissues and urine. The presence of this lipid class, members of which possess marked chelating properties, adequately accounts for the well-documented antiallergic activity of tissue and urine extracts on isolated smooth muscle preparations and in guinea pigs in vivo. This review summarizes the investigations leading to the discovery of this lipid class. It is postulated that the observed antiallergic activity of 2,4-diketones may be explained by the intracellular sequestration of calcium ions transiently liberated from intracellular storage pools after an agonist–receptor interaction, thus blocking response to the agonist. The true physiological function (or functions) of this lipid class has not been defined. The chelating properties of 2,4-diketones suggest their involvement in intracellular ionic interactions. —**Douglas, D. E.** Higher aliphatic 2,4-diketones: a ubiquitous lipid class with chelating properties, in search of a physiological function. *J. Lipid Res.* 1991. **32:** 553–558.

Supplementary key words tissue antiallergic principle • gas chromatography • chelating agents • sequestration of intracellular Ca^{2+}

For almost four decades it has been recognized that an antiallergic principle is present in mammalian tissues and in urine. It was first shown by Kovacs (1) that extracts of leucocytes had antihistamine properties. Shortly thereafter, Karady et al. (2) reported that ether–chloroform-extracted material prepared from various animal tissues and from human urine inhibited the action of histamine on smooth muscle preparations. These findings were subsequently amply confirmed (3–11), and it appeared that, to a greater or lesser degree, all mammalian soft tissues and urine contain an antihistamine principle. Such extracts also possessed antiserotonin, antibradykinin, and antiacetylcholine activity, inhibited the Schultz-Dale reaction, and protected sensitized guinea pigs against anaphylactic shock (4). It was noted that, after stress, the urinary excretion of this principle was markedly increased (2, 7, 11). Tissue concentrations of the principle varied from one individual to another, and in pathological as compared with normal tissue. In human tumor tissues, it was reported that there was, in general, a greater amount of antihistamine activity than in normal tissue adjacent to the tumours (5). Kovacs and Voith (12) found that urine extracts containing the antihistamine principle antagonized histamine-induced gastric hypersecretion in guinea pigs and hindered or prevented the development of experimentally induced gastric ulcer formation in rats. Since the bioassay methods that were used were not specific, it was not known if the same substance was responsible for the observed phenomena in all cases. It was believed by some investigators that the active principle might be an unidentified steroid. The elucidation of the chemical identity of this principle was, therefore, of great interest and of potential importance.

In 1958, Francis and Melville (13, 14), while investigating the etiology of gingival hyperplasia associated with Dilantin (diphenylhydantoin) therapy, reported the detection of a histamine-inhibiting substance in acid hydrolysates of gingival tissue specimens prepared for the histamine bioassay procedure of Barsoum and Gaddum (15). It was not known at that time whether or not this gingival antihistamine was identical to the principle found in other tissues. Our investigations, extending over some 15 years, and which are reviewed in this communication, confirmed the common identity of the gingival antihistamine with the principle found in all other tissues and in urine as a homologous series of aliphatic 2,4-diketones with chain lengths from C_{13} to C_{25} (16). Diketones with monounsatured chains may also occur. The widespread presence of these compounds, which possess versatile and strong chelating ability, suggests their involvement in intracellular ionic processes. No later publications on tissue 2,4-diketones have appeared in the literature than ours,

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and consequently, their physiological function remains unknown.

EARLY INVESTIGATIONS

It was observed that the gingival histamine inhibitor was extractable from HCl-tissue hydrolysates by solvents such as chloroform, diethyl ether, or ethyl acetate. When solvent-extracted material or samples prepared for bioassay were chromatographed on paper (Whatman no. 1, descending) with the upper phase of n-butanol–acetic acid–water 4:1:5, maximum inhibitory activity coincided with a region at R_f 0.86. This region gave a positive reaction for C=O with 2,4-dinitrophenylhydrazine, but failed to react with reagents of imidazole, phenols, or primary amino groups. The chromatographic behavior of this component led us to assume that it was not a simple aldehyde or ketone, but that it must also contain a polar group, such as hydroxyl (17). The presence of –OH as well as C=O was later confirmed by infrared spectrophotometry of purified material.

To obtain larger quantities of the inhibitory substance, tissues other than gingiva—human colon, breast, stomach and lung and bovine lung—were assayed by the procedures just described. With every sample that demonstrated antihistamine activity, the C=O-positive area was present on developed chromatograms of the extracts. Bovine lung and other animal tissues were therefore chosen as a source of the antihistamine substance.

CHEMICAL INVESTIGATIONS

Although various animal tissues were shown to contain the inhibitory substance, its isolation, free from contaminating lipids, in sufficient quantities for our chemical studies, presented difficulties initially. Thin-layer chromatography with silica gel G plates was unsuccessful.[2] However, the publication by Osman and Barson (18), in 1967, of a modified Girard T procedure in which hydrazone formation took place in dimethyl sulfoxide, provided a method that, with minor modifications, enabled us to isolate a ketonic fraction from extracts of tissue hydrolysates, free from fatty acids and other lipids (16). Further purification by chromatography on a Florisil column yielded an almost colorless oil. Typical yields of the ketonic fraction are summarized in **Table 1**.

TABLE 1. Yields of the ketonic lipid fraction from various biological samples

Type of Sample	Yield of Ketonic Fraction
	mg/kg wet weight
Beef liver	43.4
Pork liver	42.0
Beef kidney	18.3
Human urine (pooled)	0.28[a]
Human blood	7.93[a]
Human placenta	41.5

[a]Milligram/liter.

It was found that this ketonic fraction had the properties of a β-diketone. The presence of hydroxyl absorption by infrared spectroscopy, to which reference was made previously, was adequately explained by keto-enol tautomerism. Although apparently chemically homogeneous by paper chromatography, mass spectrometry indicated that this material was a complex mixture.

Until the late 1960s, when gas–liquid chromatography and appropriate stable, nonpolar silicone stationary phases became available, progress could not be made in determining the exact chemical nature of this material. On a column packed with 10% UCW98 on Diatoport S, the ketonic material was resolved into ten or more peaks, most of which were found to belong to a homologous series, ranging from C_{13} to C_{25}. These proved to be higher homologues of 2,4-pentanedione (acetylacetone).

To identify these components, fourteen saturated aliphatic 2,4-diketones from C_7 to C_{25} and one unsaturated one, Δ^{12}-2,4-heneicosenedione, were synthesized (19). In addition to the comparison of relative retention times with these standards, individual components were isolated by preparative gas–liquid chromatography, and their identities were further confirmed by *a*) comparison of their methoxime derivatives with authentic standards; *b*) the identification of fatty acid and acetone after alkaline hydrolysis; and *c*) permanganate–periodate oxidation to a monocarboxylic acid, or, in the case of a monounsaturated component, to a monocarboxylic and a dicarboxylic acid. Details of these procedures are described elsewhere (16, 19). Unfortunately, neither the techniques of gas–liquid chromatography–mass spectrometry nor high pressure liquid chromatography were available at the time our investigations were in progress. The following were unequivocally identified: 2,4-heptadecanedione, 2,4-nonadecanedione, 2,4-heneicosanedione and Δ^{12}-2,4-heneicosenedione. 2,4-Docosanedione was identified by mass spectrometry (16). 2,4-Tridecanedione and 2,4-pentadecanedione were confirmed via their methoximes (16). Typical gas–liquid chromatographic profiles are illustrated in **Fig. 1** and **Fig. 2**.

[2]The reason for our lack of success with this technique became obvious later, when the β-diketone nature of the extracts was discovered. Often, the inhibitory substance became inactivated during chromatography because of chelation with the calcium of the binder incorporated in the silica gel G. Silica gel H films, which do not contain calcium sulfate binder, were used later (16).

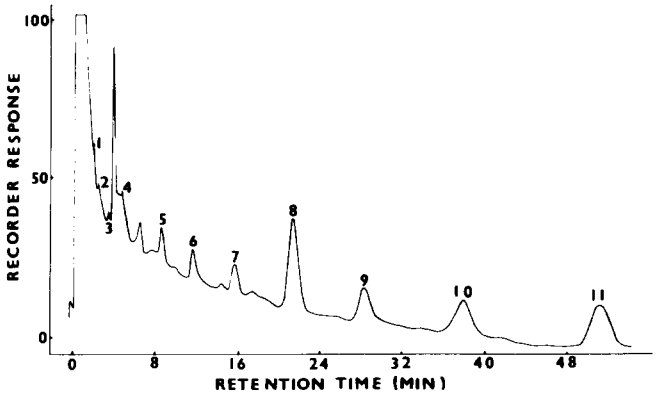


Fig. 1. Gas-liquid chromatogram of ketonic lipid extract prepared from beef brain by the Girard T. procedure. The amount of extract injected was isolated from 34 g (wet weight) of tissue (column 10% UCW 98; temperature 230°C; sensitivity, 1/2000). Component 2,4-diketones were identified as follows: 1, C_{13}; 2, C_{14}; 3, C_{15}; 4, C_{16}; 5, C_{18}; 6, C_{19}; 7, C_{20}; 8, C_{21}; 9, C_{22}; 10 and 11, not identified. Reprinted from *Can. J. Biochem.* 1978. **56:** 695 by permission.

That a part, at least, of the 2,4-diketone fraction apparently exists in tissues in a free, or labile, state was shown by the isolation of this fraction from a lipid extract of a sample of bovine liver by copper chelate formation, thus avoiding an acid hydrolysis step (16). Although other investigators used direct solvent extraction of homogenized tissues, we routinely carried out a preliminary acid hydrolysis of tissue specimens. We observed that this 2,4-diketone lipid fraction was present primarily intracellularly; it was not detected in human blood plasma or seminal plasma, but it was present in whole blood and in pooled spermatozoa.

In in vivo experiments in which guinea pigs were exposed successively to histamine aerosol and then to an aerosol containing individual synthetic 2,4-diketones or mixtures of these, the ability of these compounds to counteract the effects of histamine anaphylaxis was demonstrated (20). The histamine-blocking activity of these compounds in experiments with the guinea pig ileum preparation was similar to the previously reported activity of crude tissue extracts (17). In both the in vivo and in vitro studies, the potency of homologous 2,4-diketones increased with increasing molecular weight. Thus, 2,4-nonadecanedione was more potent than the C_{14}, C_{15}, or C_{17} homologues in the in vivo experiments, and 2,4-tridecanedione was the most active in vitro of the C_7, C_9, and C_{13} homologues.

DISCUSSION

The investigations that have been summarized have resulted in the identification of the antiallergic principle, which is apparently ubiquitously present in human and mammalian tissues and in urine, as a group of higher aliphatic 2,4-diketones. They appear to be formed in vivo by the condensation of a molecule of fatty acid with a molecule of acetone, apparently by an enzymatic mechanism. The actual pathway is unknown, but the overall reaction may be summarized as follows:

$$RCOOH + CH_3COCH_3 \rightarrow RCOCH_2COCH_3$$

where $R = CH_3(CH_2)_n$ and $n = 8$–20.

Several peaks that could not be identified by their relative retention times were frequently present in gas chromatograms of tissue extracts; these might represent unsaturated or branched-chain compounds. Since authentic 2,4-diketone standards with polyunsaturated chains were unavailable, it was not possible to determine whether such compounds were present in our extracts. Although Δ^{12}-2,4-heneicosenedione was identified, it was not always present. It is possible that the enzymatic process may favor the formation of saturated compounds, or that compounds with polyunsaturated chains, if present initially in tissue specimens, might have been lost during the work-up procedure. Components of greater chain lengths than C_{25} might not elute from the chromatographic column under the conditions that were used.

Perhaps of greater significance than the identification of the physiological antihistamine principle is the discovery of this lipid class. It is not surprising that this group of compounds previously evaded detection, because of its presence in low concentration in tissues (ca. 0.001–0.005%), and because of the facile conversion of 2,4-diketones to fatty acids and acetone in the presence of base. Moreover, since these compounds are always pres-

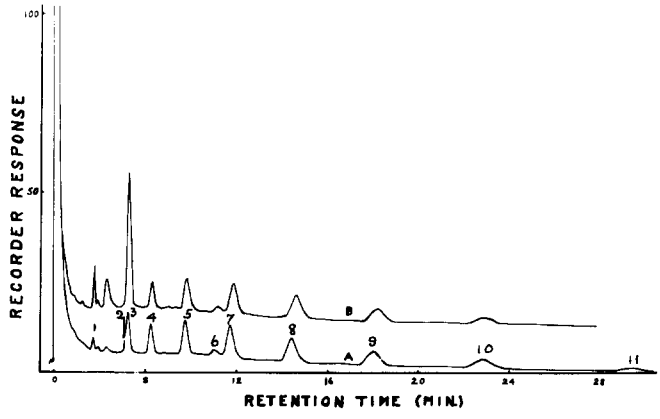
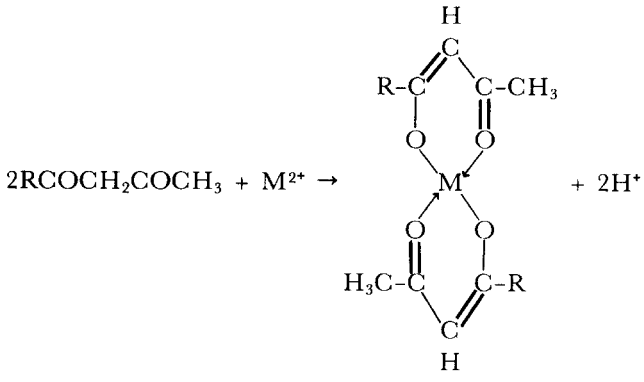


Fig. 2. A. Gas-liquid chromatogram of a ketonic lipid extract from human blood. B. The same with added internal standard 2,4-heptadecanedione (0.25 μg); the latter eluted just before peak 3. The amount of extract in each injection was from approximately 72 ml of blood (column 10% UCW 98; temperature 230°C, sensitivity 1/320). Component 2,4-diketones were identified as follows: 1, C_{15}; 2, C_{17}; 3, unidentified; 4, C_{18}; 5, C_{19}; 6, unidentified; 7, C_{20} (interpolated); 8, C_{21}; 9, C_{22} (interpolated); 10, 11 unidentified.

ent in biological materials as a complex mixture, prior resolution of the mixture into individual components would be essential before positive identification could be made. Our investigations and those of others were confined to human and mammalian issues; therefore, it is not known whether 2,4-diketones are present also in the tissues of other animal species.

2,4-Pentanedione (acetylacetone) and 2,4-diketones in general are notable for the ease with which they form thermodynamically stable chelates with practically all di- and polyvalent metal cations, including the alkaline earths (21–23). β-Diketonates of the alkali metals are also known (24). There is a voluminous literature on the physical and chemical properties and structures of metal diketonates. Chelation takes place through the binding of a metal ion to one (monovalent cation), two (divalent cation), or three (trivalent cation) ligand 2,4-diketone molecules, one, two, or three protons, respectively, being eliminated in the reaction. Both the primary (ionizable) and the secondary (nonionizable) valencies of the metal are involved in the complex (21):



(R = $CH_3(CH_2)_n$ and M is a divalent metal atom).

These chelates are stable at neutral pH, tend to be lipid-soluble, and at a low pH they are reconverted to 2,4-diketones and metal cations.

Several circumstances facilitated our investigations. Gingival tissue, on which our original observations were made, is practically devoid of fat, and hence the acid hydrolysates of samples of this tissue could be chromatographed directly on filter paper. Moreover, the active principle could be extracted with solvent without gross contamination with other lipids. Our early recognition that the inhibitory substance was a polar carbonyl-containing substance was further substantiated by our unexpected finding that an unidentified by-product of the alkaline hydrolysis of 2-bromoheptaldehyde to 2-hydroxyheptaldehyde behaved in the same manner in vitro and in vivo as did tissue extracts (17).

The isolation of carbonyl compounds from biological materials has posed many difficulties for investigators in the past, as is evidenced by the numerous publications in this area. Fortunately, the Girard T procedure described by Osman and Barson (18), which they had used for the isolation of carbonyl compounds from cigar smoke, proved to be invaluable in the fractionation of the lipids extracted from tissue hydrolysates. Gas–liquid chromatography, with the appropriate low polarity, heat-stable silicone gum stationary phases, such as UCW98 and OV-1, possessed the requisite resolving power to separate the carbonyl fraction into its many components. Great care was exercised to free the final ketonic extracts from traces of metal ions with disodium EDTA, and to prevent the subsequent contamination of the extracts before injection into the gas chromatograph.

Since the 2,4-diketones are present in urine, and are avid chelating agents, it was not surprising that they were detected in samples of individual or pooled renal calculi which we examined (25). They are probably present in the matrix as calcium and magnesium chelates.

At the time of the identification of this lipid class, we were not able to furnish a logical explanation for its remarkable and nonspecific antiallergic properties. Pharmacologically, the 2,4-diketones, being chemically unrelated to histamine and the other agonists mentioned, are not true histamine antagonists. Their inhibitory properties must, therefore, involve a mechanism other than competition with agonists at cellular receptor sites. Inactivation of histamine through Schiff base formation seemed improbable, as did the chemical alteration of surface receptors (26), since the 2,4-diketone inhibition of an agonist-induced contraction of a smooth muscle preparation was reversible; the preparation slowly regained its initial sensitivity to the agonist.

In early unpublished in vitro studies with guinea pig ileum preparations, we found that the chelating function was necessary for inhibitory activity; conversion of a 2,4-diketone to the calcium chelate essentially eliminated its inhibitory activity against histamine-caused contractions. Diketones other than β-diketones were inactive or only weakly inhibitory, and α-keotaldehydes irritated the ileum or caused a toxic reaction. Moreover, salicylaldehyde, a known chelating agent, was found to inhibit histamine in vitro. 2,4-Pentanedione, the lowest 2,4-diketone homologue, surprisingly, was inactive. However, a more recent study (20), to which reference has already been made, has shown that increasing chain length in the 2,4-diketone series above C_7 conferred increasing inhibitory potency against histamine both in vitro and in vivo (20).

As a result of progress in succeeding years in the understanding of the importance of intracellular Ca^{2+} (Ca_i^{2+}) as a trigger in the initiation of a multitude of physiological responses to external stimuli, either alone or in close association with calmodulin, troponin C, or other Ca^{2+}-binding proteins (27), an explanation for the antiallergic properties of 2,4-diketones may now be proposed.

In particular, it is now known that the interaction of an agonist at a cellular smooth muscle receptor site initiates a chain of events, which includes the induction of phosphatidylinositol 4,5-biphosphate hydrolysis to diacylglycerol and inositol 1,4,5-triphosphate. This latter compound, a second messenger, liberates Ca_i^{2+} from intracellular pools (28), resulting in a transient increase in $[Ca_i^{2+}]$. The Ca_i^{2+}, in combination with calmodulin, mediates the enzymatic phosphorylation of myosin, eventually resulting in a contraction. Should either Ca_i^{2+} or calmodulin be unavailable, smooth muscle contraction cannot occur. On the basis of this presently accepted theory of Ca_i^{2+} as the trigger of intracellular processes, and with the evidence of our observations cited above, we now postulate that after an agonist–receptor interaction in the presence of added 2,4-diketone, the transient increase in $[Ca_i^{2+}]$ that would normally occur, is prevented by its rapid sequestration, and thus smooth muscle contraction is inhibited. As the 2,4-diketones are neutral molecules, at least in their keto-form, and possess low molecular weights, it may be assumed that they can pass freely across cell membranes. This mechanism also accounts for the nonspecificity with respect to the agonist of 2,4-diketone inhibition. If this postulated mechanism is valid, other physiological cellular functions, such as cell proliferation, secretion, etc., which are Ca^{2+}-dependent, should likewise be inhibited by 2,4-diketones. The widespread occurrence of 2,4-diketones suggests that they might have been present in the extracts of urine and of various tissues that were reported by earlier investigators to possess growth-inhibitory and other biological properties (29, 30).

The stress-induced enhanced urinary excretion of the antihistamine principle (i.e., the 2,4-diketone lipid fraction) (7) may be of significance in the reaction of the organism to stress. In a study of biochemical parameters that could be used to assess the fitness of patients to undergo serial surgery, C. J. Umberger may have isolated this lipid fraction. He noted that, in severe trauma cases, an unidentified ether-extractable substance appeared in the patients' urine, possessing an absorbance maximum at 270 nm, (the region of maximum absorption of β-diketones) and that, during recovery, the excretion of this component decreased (the late Dr. C. J. Umberger, private communication, 1959). The observation of Pelletier, Kovacs, and Rose (5) that higher antihistamine activity was found in tumor tissue specimens than in normal tissues may be of significance in oncogenesis.

It is well known that chelates and chelation play essential roles in numerous biological systems, and that chelation is of importance in the pharmacological activity of many drugs. As the picture of intracellular ionic processes continues to unfold, it is the author's hope that the true physiological function of 2,4-diketones in the life process may be defined. The deaths of both Drs. K. I. Melville and L. E. Francis in the early 1970s terminated our investigations. In retrospect, lacking the present knowledge of cellular physiology, especially with respect to the importance of intracellular Ca^{2+}, and without the availability of modern techniques for the study of intracelllular biochemistry, it would have been futile for us at that time to attempt to elucidate the physiological function of 2,4-diketones. The presently postulated ability of these compounds to by-pass agonist–receptor responses through direct inhibition of intracellular Ca^{2+} flow justifies further investigation into the pharmacology of these interesting compounds and of their analogues and derivatives, which may well lead to useful therapeutic applications.[3]

It is notable that the serendipitous identification of an interfering material in the bioassay of histamine in gingival tissue has resulted in the discovery of this 2,4-diketone lipid class, a finding that is probably of greater scientific significance than the elucidation of the etiology of diphenylhydantoin-induced gingival hyperplasia, which was the original research project.

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