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Uncoupling of oxidative phosphorylation by glycyrrhetic acid, fusidic acid and some related triterpenoid acids

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Some new derivatives of 18α- and 18β-glycyrrhetic acid and oleanolic acid were tested for their ability to inhibit phosphorylation coupled to succinate oxidation in rat liver mitochondria. Glycyrrhetic acid is a potent uncoupler of oxidative phosphorylation (approaching 2,4-dinitrophenol in potency); uncoupling activity is dependent upon each of the following functional groups: 3-hydroxy, 11-oxo and 30-carboxyl groups. Inversion of the configuration at C-18 (D/E ring junction) or replacement of the 11-oxo-12-ene system in ring C by the 9(11),12-diene system in glycyrrhetic acid abolished uncoupling activity. By contrast, the hemisuccinates (3-*O*-carboxypropionyl derivatives) of 18α-glycyrrhetic acid and of the 18β-9,12-diene acid were moderately potent uncoupling agents but less active than 18β-glycyrrhetic acid. The uncoupling activity of these hemisuccinates could not be attributed to the carboxypropionyl group alone. Structural analogues of various glycyrrhetic acid derivatives prepared from oleanolic acid (with the carboxyl group at C-28) were less active in uncoupling oxidative phosphorylation than the corresponding compounds in the glycyrrhetic acid series (with the carboxyl group at C-30). The uncoupling activity of some derivatives of two naturally occurring tetracyclic triterpenoid acids, polyporenic acid A and fusidic acid, was also investigated and found to largely depend upon their chemical structure. The possible application of these compounds as drugs in man is discussed.

GLYCYRRHETIC acid (glycyrrhetinic acid, Fig. 1A), the aglycone of glycyrrhizin (from liquorice) uncouples oxidative phosphorylation, that is, it inhibits the mitochondrial biosynthesis of adenosine 5′-triphosphate without inhibiting mitochondrial respiration.

Polyporenic acid A (ungulinic acid) is a tetracyclic triterpenoid (trimethyl sterol), one of several polyisoprenoids produced by the birch fungus *Polyporus betulinus* (Jones & Halsall, 1954; Fieser & Fieser, 1959; Ourisson, Crabbe & Rodig, 1964). It is 3α,12α-dihydroxy-24-methylene-5α,25ξ-lanost-8-en-26-oic acid (Fig. 1B) (Halsall, Hodges & Jones, 1953; Halsall & Hodges, 1954). Polyporenic acid A uncouples oxidative phosphorylation in liver mitochondria (Whitehouse, 1963) but does not exhibit anti-inflammatory activity in rats when assayed by the carrageenan granuloma test (private communication from Dr. R. M. A. Atkinson, Glaxo Laboratories).

Fusidic acid (Fusidin) is an antibiotic originally obtained from *Fusidium coccineum* (Godtfredsen, Roholt & Tybring, 1962; Newman, Bhat & others, 1962) and subsequently shown to be identical with ramycin, isolated from several cephalosporia and a phycomycete (Vanderhaeghe, Van Dijck & De Somer, 1965). Most features of its structure have now been elucidated (Fig. 1C) (Godtfredsen & Vangedal, 1962; Arigoni,

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Daehne & others, 1963; Bucourt & Legrand, 1964; Godtfredsen, Daehne & others, 1965). It has recently been found to alleviate arthritis in man (Dr. W. O. Godtfredsen, private communication).

We have now examined the relationship between chemical structure and uncoupling activity of several derivatives and analogues of glycyrrhetic acid, polyporenic acid A and fusidic acid.

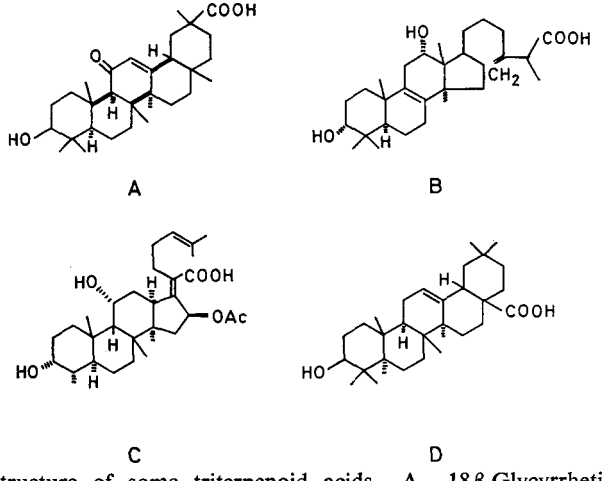

FIG. 1. Structure of some triterpenoid acids. A. 18β-Glycyrrhetic acid. B. Polyporenic acid A. C. Fusidic acid. D. Oleanolic acid.

Experimental

Materials. The following compounds were kindly made available by Dr. S. Gottfried & the late Prof. E. E. Turner, F.R.S. (Biorex Laboratories, London, E.C.1): oleanolic acid, purified 18α- and 18β-glycyrrhetic acid, the corresponding 3-*O*-hemisuccinate (β-carboxypropionyl) esters, the 3-*O*-sulphate ester (as the Na salt) of the 18β-acid, 3-dehydro- and 11-deoxo-18β-glycyrrhetic acid, 3-*O*-acetyl- and 3-*O*-propionyl-18β-glycyrrhetic acid, certain conjugates of oleanoic acid and glycyrrhetic acid with anthranilic acid and *p*-aminobenzoic acid, and a highly purified preparation of glycyrrhizin (originally obtained from Ihei Matsumoto & Co., Ltd., Tokyo). A commercial sample of ammonium glycyrrhizate was obtained from L. Light & Co., Colnbrook, Bucks.

Other derivatives of the glycyrrhetic acids and derivatives of oleanolic acid were synthesized as described (Dean, Halsall & Whitehouse, 1967).

Polyporenic acids and their derivatives were prepared according to Curtis, Heilbron & others (1953); Jones & Woods (1953) and Halsall & Hodges (1954). Eburicoic acid was isolated from *Polyporus eucalyptorura*. 3β-Hydroxycholest-5-en-26-oic acid was synthesized from 25-oxonorcholesterol (Dean & Whitehouse, 1966). Fusidic acid and some of its derivatives (Godtfredsen & Vangedal, 1962) were supplied by Dr. W. O. Godtfredsen (Leo Pharmaceutical Products, Copenhagen). Tumulosic acid was donated by Dr. J. E. E. Holker, University of Liverpool and cholesterol 3-*O*-(hydrogen sulphate) by Mr. P. R. H. Raggatt.

METHODS

Rat liver mitochondria were isolated and incubated at 30° with various oxidizable substrates, glucose and yeast hexokinase at pH 6·8 as detailed by Skidmore & Whitehouse (1965a). Oxygen uptake was measured manometrically; phosphate uptake was measured colorimetrically (Fiske & Subbarow, 1925). Compounds to be tested for their effect upon oxidative phosphorylation were added to the main compartment of Warburg vessels either in 100 μl of neutralized aqueous solution or in 25 μl of dimethylformamide together with the theoretical equivalent of aqueous sodium bicarbonate.

The activity of yeast hexokinase as a glucose phosphorylase in the presence of these triterpenoids was determined by the method of Darrow & Colowick (1955).

Drug binding to ϵ-amino-groups of bovine plasma albumen was determined colorimetrically using 2,4,6-trinitrobenzaldehyde (Skidmore & Whitehouse, 1965b; 1966); *N*-benzoyl-L-arginine ethyl ester was substrate.

Drug action on a thiol enzyme (papain) was studied by Whitehouse & Leader (1967).

Results

Glycyrrhetic acid (100 μM) and polyporenic acid A (200 μM) were fairly potent uncouplers of oxidative phosphorylation. A higher concentration of fusidic acid (600 μM) was needed for the same effect *in vitro*. At these concentrations these triterpenoid acids abolished the phosphorylation of ADP (i.e. uptake of inorganic phosphate) accompanying the mitochondrial oxidation of succinate, pyruvate, glutamate and citrate. Also, they had no effect on mitochondrial respiration or on either the glucose-phosphorylating or the ATP-ase activity (de la Fuente & Sols, 1963) of the yeast hexokinase which was added, with glucose, to the phosphorylating mitochondrial preparations to trap the newly incorporated labile γ-phosphate group of ATP (as the stable ester-phosphate group in glucose 6-phosphate). The uncoupling action of these acids was not reversed by co-incubation with 5 mM cysteamine or mercaptoethanol but was reversed on diluting the drug after preincubation with mitrochondria.

Further evidence that these acids were uncouplers of oxidative phosphorylation was found as follows. Slices of cattle tracheal cartilage were incubated with sodium [^{32}P]phosphate and with sodium [^{35}S]sulphate and the incorporation of ^{32}P into organic phosphates and the ATP-dependent incorporation of ^{35}S into the polysaccharide sulphates of the cartilage tissue were each determined in the presence and in the absence of these triterpenoids (Whitehouse & Haslam, 1962; Whitehouse, 1964a). Glycyrrhetic acid (100 μM) and polyporenic acid A (200 μM) each inhibited these biosynthetic processes by more than 50% without impairing the oxidation of [$^{14}C_U$]glucose and [$^{14}C_1$]octanoate to [^{14}C]carbon dioxide by these cartilage slices. Oleanolic acid (300 μM) and 11-deoxo-18β-glycyrrhetic acid (comp. 12; Table 1) (150 μM), which do not uncouple oxidative phosphorylation in liver mitochondria (see below), had no effect on either ^{32}P or ^{35}S incorporation by cartilage slices.

The relationship of chemical structure to uncoupling activity was explored with selected derivatives of glycyrrhetic, oleanolic, polyporenic and fusidic acids, using rat liver mitochondria incubated with succinate as the test system. A compound which lowered the P/O ratio to $\leqslant 85\%$ that of the P/O ratio obtained in parallel incubations without added triterpenoids, was considered to be capable of uncoupling oxidative phosphorylation. The P/O ratio in these drug-free controls (assigned a value of 100%) actually varied with each mitochondrial preparation but fell within the range 1·2–1·7 (see Skidmore & Whitehouse, 1965a).

With this biological test system, a fairly reproducible response was obtained to a given concentration of a triterpenoid. Thus in duplicate assays for 75 μM glycyrrhetic acid with 20 successive mitochondrial preparations, succinate oxidation was inhibited by no more than 20% and the P/O ratio was 32 $\pm$ 7 (s.e.m.)% of the controls; the range of P/O values being actually 10–55% of the controls.

UNCOUPLING ACTIVITY OF GLYCYRRHETIC ACID DERIVATIVES

Table 1 indicates the relative uncoupling potencies of 18β-glycyrrhetic acid and 17 of its derivatives. Simple derivatives lacking the ionized carboxyl group at C-30 were too water-insoluble for testing. Several

TABLE 1. UNCOUPLING ACTIVITY OF SOME DERIVATIVES OF 18α- AND 18β-GLYCYRRHETIC ACID AND RELATED COMPOUNDS

No.	Compound	Conc. $\times 10^{-4}$M	% Control P/O ratio
—	None	—	100
1	18α-Glycyrrhetic acid(isoglycyrrhetic acid)	1·5	92
2	18β-Glycyrrhetic acid	0·75	35
		1·5	5
3	3-Dehydro-18β-glycyrrhetic acid	0·75	83
4	Methyl 18β-glycyrrhetate	1·0*	86
5	3-*O*-Acetyl-18β-glycyrrhetic acid	0·75	73
		1·5	38
6	3-*O*-Propionyl-18β-glycyrrhetic acid	0·75	85
7	3-*O*-(β-Carboxypropionyl)-18α-glycyrrhetic acid	2·2	0
		1·2	43
8	3-*O*-(β-Carboxypropionyl)-18β-glycyrrhetic acid	2·2	34
		1·2	70
9	Methyl 3-*O*-(β-carboxypropionyl)-18α-glycyrrhetate	1·2	84
10	Methyl 3-*O*-(β-carboxypropionyl)-18β-glycyrrhetate	1·5	100
11	3-*O*-Acetyl-18β-glycyrrhetamide	0·75	100
12	11-Deoxo-18β-glycyrrhetic acid	1·5	96
13	3-*O*-(β-Carboxypropionyl)-11-deoxo-18β-glycyrrhetic acid	2·0	80
14	9,11-Dehydro-11-deoxo-18β-glycyrrhetic acid	0·75	100
15	3-*O*-(β-Carboxypropionyl)-9,11-dehydro-11-deoxy-18β-glycyrrhetic acid	2·0	5
		1·0	25
16	Glycyrrhizin (purified Na salt)	7·5	92
17	Glycyrrhizin (commercial NH_4^+ salt)	5·0	74
18	18β-Glycyrrhetic acid 3-*O*-(hydrogen sulphate)	2·5	0–70†
19	*N*-(18β-Glycyrrhetyl)glycine	1·5	65
20	*o*-(18β-Glycyrrhetamido)benzoic acid	1·5	53
21	*o*-[3-*O*-(β-Carboxypropionyl)-18β-glycyrrhetamido]benzoic acid	0·75	55
22	*o*-(3-*O*-Acetyl-18β-glycyrrhetamido)benzoic acid	0·75	30
		0·4	55
23	*p*-(3-*O*-Acetyl-18β-glycyrrhetamido)benzoic acid	0·75	95
24	*o*-Cholamidobenzoic acid	4·0	45
25	Cortisone 21-sulphate (Na salt)	2·5	98
26	Cholesterol 3-*O*-(hydrogen sulphate) (K salt)	2·5	100

* Conc. uncertain due to limited solubility.
† Variable activity, see text.

other derivatives, which retained this carboxyl group, were too insoluble in the presence of magnesium ions (4·5 mM) at pH 6·8 to be tested adequately. In either instance, when this happened with compounds containing a 3β-hydroxyl group, it was usually possible to test their 3-*O*-(β-carboxypropionyl derivatives ("hemisuccinate esters") for uncoupling activity instead, these particular derivatives being much more soluble than their parent alcohols in aqueous media containing magnesium ions.

3-*O*-(β-Carboxypropionyl)-18β-glycyrrhetic acid (comp. 8) was approximately one-third as potent as uncoupling agent as the parent alcohol, i.e. 18β-glycyrrhetic acid (comp. 2). The corresponding hemisuccinate of 18α-glycyrrhetic acid (comp. 7) was more active in uncoupling oxidative phosphorylation than the hemisuccinate of the acid (comp. 8). By contrast, the parent alcohol (comp. 1) was inactive; it was certainly sufficiently water-soluble in the presence of magnesium ions at pH 6·8 to be compared with the unsubstituted 18β-acid (comp. 2). In two other instances it was likewise found that hemisuccinates uncoupled oxidative phosphorylation (comp. 13 and 15), but the corresponding 3-hydroxy compounds did not (comp. 12 and 14).

Some "quasi-physiological" partition experiments were made in attempts to find an explanation for these findings. The removal of ultraviolet absorbing 3-hydroxy compounds and hemisuccinates from the aqueous salt solution pH 6·8 by liver mitochondria during a 30 min incubation at 30° was measured as follows. The residual ultraviolet absorption at 250 mμ [due to the 11-oxo-12-ene and the 9(11),12-diene chromophores respectively] in the aqueous phase was determined after removal of the mitochondrial lipid phase (at 8,500 *g*, 15 min) and subsequent partition (concentration) of the ultraviolet absorbing triterpenoids into methylene dichloride at pH 6·8; parallel assays were made on solutions of the triterpenoids similarly incubated without mitochondria.

These experiments showed that the parent 18α- and 18β-acids were both removed from the aqueous phase to approximately the same extent (ca. 80%) by thick mitochondrial suspensions (ca. 20% by volume); only 20% of the hemisuccinate of the 18α-acid was removed under the same conditions. Whilst these results indicate why the uncoupling activity of the hemisuccinate of the 18β- acid was only 1/4 to 1/3 that of the parent 18β-acid, they give no indication why the parent 18α-acid should be inactive while its hemisuccinate is a potent uncoupling agent. The hemisuccinate group itself apparently had no uncoupling activity; at least 5 of the other hemisuccinates of triterpenes tested had no effect upon oxidative phosphorylation (see below).

Replacement of the ionized carboxyl group at C-30 effectively abolished uncoupling activity; the methyl esters of 18β-glycyrrhetic acid (comp. 4) and of the hemisuccinates of 18α- and 18β-glycyrrhetic acids (comp. 9 and 10) and the amides of both the 18β-glycyrrhetic acid and the 3-*O*-acetyl-18β-acid (comp. 11), were each without any effect on oxidative phosphorylation [18β-glycyrrhetamide was not pure: it was tested in admixture with methyl glycyrrhetate (which is inactive) from which it was prepared by ammonolysis in liquid ammonia; the amide has a higher

Rf value than the ester on thin-layer plates developed with ethyl acetate].

Other more soluble derivatives of glycyrrhetinamide were also examined for uncoupling activity. Only the *N*-anthranilic acid (comp. 20) and *N*-glycine conjugates (comp. 19) had any significant activity. Esterification of the 3-hydroxyl group of the anthranilic acid conjugate (to give comp. 21 and 22) actually increased the uncoupling activity, perhaps by increasing the solubility.

Modification of the 3β-hydroxyl group of 18β-glycyrrhetic acid, either by oxidation to the ketone (comp. 3) or by esterification (comp. 5,6,8 and 18) diminished but did not abolish the uncoupling activity. The 3-*O*-acetyl, 3-*O*-(hydrogen sulphate) and 3-*O*-(β-carboxypropionyl) derivatives (comp. 5, 18, 8), though differing greatly in the ease with which they partition between liver mitochondria and aqueous solutions of pH 6·8 (the acetyl ester resembling the unesterified acid, see above) were all less active than the parent 3β-hydroxyl compound (comp. 2). The hydrogen sulphate showed variable uncoupling activity (relative to the parent acid; comp. 2) in different experiments, which may reflect some hydrolysis to the parent acid. This ester is labile, particularly in acidic media (Dr. J. C. Turner, private communication). The very water-soluble 3-glycoside, glycyrrhizin (comp. 16 and 17) was inactive. These observations indicate that the presence of an unsubstituted hydroxyl function at C-3, though not essential for uncoupling activity, nevertheless determines the absolute activity of this type of compound in uncoupling oxidative phosphorylation.

Removal of the 11-keto-group of 18β-glycyrrhetic acid, with concomitant reduction of C-11 to a methylene group, virtually abolished the uncoupling activity (cf. comp. 12 and 13 with comp. 2 and 8). However, when the 11-oxo-12-ene grouping in 18β-glycyrrhetic acid was replaced by the 9(11),12-diene grouping the uncoupling activity was apparently retained in one instance (cf. comp. 15 with comp. 8) but not in another (cf. comp. 14 and 2). This situation corresponded to that found with the hemisuccinate of glycyrrhetic acid (active) and the unsubstituted 18α-acid (inactive); in each instance the parent 3β-hydroxy compounds were inactive whilst the 3-carboxypropionyl esters were active.

UNCOUPLING ACTIVITY OF OLEANOLIC ACID DERIVATIVES AND OTHER PENTACYCLIC ACIDS

Oleanolic acid (comp. 27, Table 2) is an isomer of 11-deoxo-18β-glycyrrhetic acid in which the carboxyl group is at C-28 (in the junction of the D and E rings) instead of at C-30 as in the glycyrrhetic acid series.

Neither 18β-oleanolic acid nor its hemisuccinate (comp. 28) uncoupled oxidative phosphorylation (Table 2). The hemisuccinate of 11-deoxo-glycyrrhetic acid (comp. 13), though non-absorbing at 250 mμ (i.e. 11-oxo-12-ene grouping was absent) always showed some slight but significant uncoupling activity. Other analogues of active compounds in the glycyrrhetic acid series were prepared from oleanolic acid, e.g. 11-oxo-18β-oleanolic acid, for comparison with 18β-glycyrrhetic acid (Fig. 1A). These oleanolic acids (comp. 29, 30, 31, Table 2) were less potent uncoupling agents than the corresponding glycyrrhetic acids

TABLE 2. UNCOUPLING OF OXIDATIVE PHOSPHORYLATION BY SOME OLEANOLIC ACID DERIVATIVES

No.	Compound	Conc. $\times 10^{-4}$M	P/O ratio (% controls)
—	None	—	100
27	18β-Oleanolic acid	1·5	100
28	3-*O*-(β-Carboxypropionyl)-18β-oleanolic acid	3	92
29	11-Oxo-18β-oleanolic acid	1·5	83
30	3-*O*-Acetyl-11-oxo-18β-oleanolic acid	1·5	83
31	3-*O*-(β-Carboxypropionyl)-11-oxo-18β-oleanolic acid	2·2	72
32	3-*O*-(β-Carboxypropionyl)-9,11-dehydro-18β-oleanolic acid	2	15
33	3-*O*-(β-Carboxyproprionyl)11-oxo-oleanolyl (28 → 13)lactone	2	100
34	*o*-(Oleanolamido)benzoic acid	1·5	45

(comp. 1, 5, 8, 15, Table 1). These findings show that the position of the carboxyl group in this series of compounds (4 oleanolic acids and 4 glycyrrhetic acids) largely governs the uncoupling activity. The most active oleanolic acid analogue encountered in this study was the hemi-succinate of 9,11-dehydro-18β-oleanolic acid (comp. 32) which contains the 9(11),12-diene system.

The inactivity of the water-soluble lactone (comp. 33) again shows the requirement for an unsubstituted carboxyl group for uncoupling activity.

Other pentacyclic triterpenoid acids tested (ursolic, boswellic, morolic and 11-oxoboswellic acids) did not uncouple oxidative phosphorylation when tested at 150 μM.

UNCOUPLING ACTIVITY OF UNSATURATED STEROIDS AND TRITERPENOIDS

Some conjugated unsaturated ketones and water-soluble conjugated dienes were tested but found not to uncouple phosphorylation linked to succinate oxidation. Compounds tested included cholest-4-en-3-one (70 μM), 11-deoxycorticosterone (deoxycortone) (100 μM), cortisol-21-aldehyde (hydrocortisone 21-aldehyde) (200 μM), 3-β-carboxypropionyl)-ergosterol (100 μM). Uncoupling activity is evidently not associated with these unsaturated groups *per se*, in the absence of a nuclear carboxyl group. Other steroid sulphates (comp. 25, 26) did not mimic glycyrrhetic acid 3-*O*-sulphate (comp. 18) in uncoupling phosphorylation (Table 1).

UNCOUPLING ACTIVITY OF SOME TETRACYCLIC TRITERPENOID ACIDS

A few derivatives of polyporenic acid A, a βγ-unsaturated acid, were examined for uncoupling activity (Table 3). One of the αβ-unsaturated

TABLE 3. UNCOUPLING OF OXIDATIVE PHOSPHORYLATION BY SOME TETRACYCLIC TERPENOID (TRIMETHYL STEROID) ACIDS

No.	Compound	Conc. $\times 10^{-4}$M	P/O ratio (% control)
35	Polyporenic acid A	1·5	30
36	Polyporenic acid C	3	100
37	Eburicoic acid	3	100
38	Fusidic acid	3	75
39	ψ-Polyporenic acid (25-isopolyporenic acid)	5	45
40	3,12-Dehydropolyporenic acid	1·5	40
41	24,28-Dihydropolyporenic acid A	1·5	50
42	3β-Hydroxycholest-5-en-26-oic acid	1·5	55
43	Tumulosic acid	3	92

isomers ψ-polyporenic acid (comp. 39) and 24,28-dihydropolyporenic acid (comp. 41) were both less active than polyporenic acid A in uncoupling oxidative phosphorylation, indicating that the double bond between C-24 and C-28 may possibly contribute to the uncoupling activity of polyporenic acid A. The diketo-derivative (comp. 40) was less active than polyporenic acid A (cf. 3-dehydro-18β-glycyrrhetic acid and 18β-glycyrrhetic acid itself (comp. 3, 2)]. A crude preparation of the naturally occurring conjugate (glycoside?) of polyporenic acid A (containing a small quantity of the free acid) was much less potent than polyporenic acid A itself on a weight for weight basis. Unless this unknown conjugate has a molecular weight at least 5 times that of the free acid, this observation suggests that the naturally occurring form(s) of polyporenic acid A is less active in uncoupling phosphorylation than the free acid (cf. glycyrrhizin and glycyrrhetic acid).

Polyporenic acid C (16α-hydroxy-24-methylene-3-oxo-5α-lanosta-7,9 (11)-dien-20-oic acid) and tumulosic acid (3β,16α-dihydroxy-24-methylene-5α-lanost-8-en-20-oic acid), which are both also obtained from *Polyporus betulinus*, and eburicoic acid (3β-hydroxy-24-methylene-5α-lanost-8-en-20-oic acid) did not uncouple oxidative phosphorylation at concentrations at which they were soluble in the buffered salt medium.

Little variation in uncoupling activity was found when the fusidic acid derivatives described by Godtfredsen & Vangedal, (1962) were tested.

RELATIVE BINDING TO ALBUMEN

There was some correlation between the solubility of a terpenoid acid in the presence of 0·1 mM bovine plasma albumen and its uncoupling activity, for example eburicoic acid, oleanolic acid, 18α-glycyrrhetic acid and 11-deoxo-18β-glycyrrhetic acid did not bind sufficiently strongly to albumen to give 1 mM solutions but polyporenic acid A, 18β-glycyrrhetic acid and fusidic acid readily gave mM solutions in the presence of 0·1mM

TABLE 4. RELATIVE BINDING OF SOME ACIDIC POLYISOPRENOIDS TO ALBUMEN (LYSYL) AMINO-GROUPS DETERMINED COLORIMETRICALLY WITH 2,4,6-TRINITROBENZALDEHYDE. Inhibitors by 1 mM drug of colour developed when reagent (0·1 mM) reacts with bovine plasma albumen (0·1 m at pH 7·4 (0·1M phosphate), determined at 425 mμ.

No.	Compound	Inhibition %
—	None	—
2	18β-Glycyrrhetic acid	35
3	3-Dehydro-18β-glycyrrhetic acid	40
5	3-*O*-Acetyl-18β-glycyrrhetic acid	38
19	*N*-(18β-Glycyrrhetyl)glycine	23
18	18β-Glycyrrhetic acid 3-*O*-(hydrogen sulphate)	48
16	Glycyrrhizin (purified Na salt)	0
17	Glycyrrhizin (commercial NH_4^+ salt)	0
8	3-*O*-(β-Carboxypropionyl)-18β-glycyrrhetic acid	60
21	*o*-[3-*O*-(β-Carboxypropionyl)-18β-glycyrrhetamido]benzoic acid	74
7	3-*O*-(β-Carboxypropionyl)-18α-glycyrrhetic acid	70
31	3-*O*-(β-Carboxypropionyl)-11-oxo-18β-oleanolic acid	75
35	Polyporenic acid A	65
41	24,28-Dihydropolyporenic acid A	44
38	Fusidic acid	20
	3,7,12-Trioxocholanic acid	0
	Deoxycholic acid	38
	3,12-Dioxocholanic acid	32

albumen. Within this latter class of albumen-soluble acids, uncoupling activity approximately paralleled the affinity of these compounds for the albumen (lysyl) ϵ-amino-groups as indicated by their ability to prevent 2,4,6-trinitrobenzaldehyde from reacting with these amino-groups and so quenching the red colouration formed by the albumen-trinitrobenzaldehyde interaction. [A similar correlation has been found in studies of aromatic anti-inflammatory acidic drugs (Skidmore & Whitehouse, 1965b).] Table 4 shows that this albumen-binding is primarily a property of the 30-carboxyl group in the glycyrrhetic acid series but is enhanced by the 3-(β-carboxypropionyl) (hemisuccinate) and 3-*O*-sulphate groups. The 3-*O*-(β-carboxypropionyl)-18α-glycyrrhetic acid (comp. 7) which is more potent than the hemisuccinate of the 18β-acid (comp. 8) in uncoupling phosphorylation, also binds more strongly to these albumen amino-groups. 3-*O*-(β-Carboxypropionyl)-11-oxo-18β-oleanolic acid (comp. 31) binds more strongly to albumen amino-groups than 3-*O*-(β-carboxypropionyl)-18β-oleanolic acid (comp. 28) but neither of these compounds significantly uncouples phosphorylation, showing that albumen-binding is of itself no absolute guide to potential uncoupling activity, though it may provide an indication of the relative potency within a given series of compounds with intrinsic uncoupling activity.

When these uncoupling acids were co-incubated with bovine plasma albumen and phosphorylating mitochondria, it was found that 75 μM 18β-glycyrrhetic acid no longer uncoupled phosphorylation in the presence of 1 mg/ml albumen. The hemisuccinates of 18α- and 18β-glycyrrhetic acids (comp. 7, 8) and glycyrrhetic acid 3-*O*-sulphate (comp. 18) (all at 25 μM) and sodium fusidate (comp. 38) (500 μM) still retained some uncoupling activity in the presence of 5 mg/ml albumen and polyporenic acid A (120 μM) retained some activity in the presence of 2·5 mg/ml albumen, but the potency of these acids was reduced (approx. 50–70%).

Discussion

These studies have been limited by the poor solubility of many of the compounds, but it is possible to draw the following conclusions.

(1) The ability of certain triterpenoid acids to uncouple oxidative phosphorylation must now be added to the list of known biological properties of terpenes (reviewed by Martin-Smith & Khatoon, 1963). The anti-inflammatory activity of glycyrrhetic acid in small animals and the inhibition of neoplastic cell growth by the triterpenoid fractions from *Polyporus betulinus* (Utzig & Samborski, 1957; Wandokanty & Utzig, 1958) may depend upon the uncoupling of oxidative phosphorylation in the subcutaneous or neoplastic tissue by glycyrrhetic and polyporenic acid A respectively. The antibacterial activity of fusidic acid (Godtfredsen & others 1962), like that of halophenols, salicyclic acid and many natural products (antibiotics) such as gramicidin and usnic acid, may also be a consequence of its uncoupling activity. The uncoupling activity of fusidic acid exceeds that of salicylic acid (Whitehouse, 1964a) whilst that of glycyrrhetic acid actually approaches the activity of 2,4-dinitrophenol

(almost complete uncoupling at 50 μM) and certainly exceeds the uncoupling activity of the most potent steroidal (cholanic) acid of animal origin, namely deoxycholic acid (Lee & Whitehouse, 1965).

(2) The uncoupling activity (and solubility at pH 7) of these particular triterpenoid acids is dependent upon their chemical structure. It should therefore be distinguished from many of the other biological activities of terpenes which are structurally non-specific (Martin-Smith & Khatoon, 1963). This dependence of the uncoupling activity upon chemical structure distinguishes these triterpenoid acids from most aliphatic fatty acids, and possibly also arylacetic acid (Whitehouse, 1964b), the uncoupling activity of which appears to depend only upon solubility in a lipid phase and the presence of a carboxyl group.

Each of the functional groups of glycyrrhetic acid (3-hydroxy,11-oxo and 30-carboxy) apparently contributes to its uncoupling activity, the carboxyl group and double unsaturation in ring C (either an 11-oxo-12-ene or a 9(11),12-diene system) being apparently essential for this purpose. The much weaker uncoupling activity of the olean-28-oic isomers (oleanolic acid derivatives) demonstrates that the activity of the glycyrrhetic acid derivatives is not a structurally non-specific activity of a highly lipophilic acid; if this were so, the oleanolic acid derivatives and glycyrrhetic acid derivatives should be approximately equipotent. The difference in activity of 18α- and 18β-glycyrrhetic acid further emphasizes this.

Similar conclusions may be drawn from our rather more limited survey of the tetracyclic terpenoid acids. The presence of the double bond in polyporenic acid A and the 20-iso-configuration in fusidic acid may be of particular importance in determining uncoupling potency. It is remarkable that the ketones, derived by oxidation of the hydroxy-group in position 3 in glycyrrhetic acid and in position 12 in polyporenic acid A, should be so much less active than the parent alcohols. This parallels the observation that 3-, 12- (and 7-) oxocholanic acids are very much weaker uncoupling agents than the corresponding hydroxycholanic acids (Lee & Whitehouse, 1965). Oxidation of these hydroxy-acids to the corresponding ketones does not seem to alter the affinity for albumen amino-groups however.

The prospect of using triterpenoid acids as effective drugs in man (antibacterials or antirheumatic agents) is tempered by the fact that they may be strongly bound to plasma proteins *in vivo* and effectively rendered unavailable, just as we found to be the case with glycyrrhetic acid in the presence of serum albumen *in vitro*. Though this protein binding of the potential drug may be much diminished by increasing the water solubility of the triterpenoid, e.g. by β-carboxypropionylation, such modification of the molecule also diminishes or abolishes the uncoupling activity. Thus the highly water-soluble natural glycoside, glycyrrhizin, is virtually inactive. The relatively low uncoupling activity of the rather water-soluble fusidic acid (salts) and of 3-*O*-(β-carboxypropionyl)-18β-glycyrrhetic acid compares unfavourably with that of the less oxygenated and much less water-soluble polyporenic acid A or glycyrrhetic acids. This increase in water solubility must effectively lower the concentration

of the acid in, or across, lipid-rich phases such as the cell membranes and within the mitochondria themselves. Furthermore in view of the rapidity with which circulating cholanic acids and many steroid hormones are taken up and conjugated by the liver, to be subsequently excreted with the bile flow, it is likely that these higher polycyclic acids will suffer a similar fate. Parke, Pollack & Williams (1963) have already shown that 95% of a single dose of radioactive glycyrrhetic acid administered intraperitoneally to rats is excreted via the bile within 6 to 8 hr, mainly as three metabolites.

One of these has now been shown to be the 3-*O*-(hydrogen sulphate) ester of glycyrrhetic acid (Iveson, Parke & Williams, 1966). This particular metabolite (comp. 18) does uncouple oxidative phosphorylation, even in the presence of plasma albumen, although it is less potent than glycyrrhetic acid itself in the absence of albumen. The glycine congugate of glycyrrhetic acid (comp. 19) was synthesized and found to be much less potent in uncoupling phosphorylation than glycyrrhetic acid itself. This conjugate does not seem to be formed in the rat; it is actually less polar than any of the glycyrrhetic acid metabolites detected by Parke & others (1963) in rat bile (Dr. D. V. Parke, private communication).

The only compounds that were more potent uncoupling agents than glycyrrhetic acid and, therefore, of potential interest as novel anti-inflammatory drugs, were the conjugates with anthranilic acid (comp. 20, 21, 22). That this was largely due to the anthranyl pharmacophore rather than the glycyrrhetyl moiety was indicated by (*a*) the feeble uncoupling activity of the analogue conjugated with *p*-aminobenzoic acid (comp. 23) and (*b*) the uncoupling activity of other *N*-acylanthranilic acids (Leader & Whitehouse, 1966; Whitehouse, 1964a and unpublished observations), including *o*-(oleanolamido)benzoic acid [*N*-(oleanoyl)anthranilic acid, comp. 34].

All these terpenoid acids appear to uncouple phosphorylation by interaction with key lysyl amino-groups participating in mitochondrial phosphorylation. There was a correlation between their ability to bind to these same groups on plasma albumen and their uncoupling potency *in vitro*. Neither the hemisuccinates of 18α- or 18β-glycyrrhetic acid (comp. 7, 8) nor fusidic acid inhibited papain (a thiol enzyme), when tested at 1 mM in the presence of 0·25 mM mercaptoethanol; the uncoupling activity of these acids and polyporenic acid A was not reversed by thiols, so it is unlikely that they uncouple phosphorylation merely by interaction with essential thiol groups in mitochondria. [This is an alternative mechanism of uncoupling phosphorylation and explains the uncoupling activity of ninhydrin (Whitehouse & Leader, 1967)].

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