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INHIBITION OF BILIARY SECRETION BY ICTEROGENIN AND RELATED TRITERPENES—II.

EFFECT OF ICTEROGENIN, CRUDE REHMANNIC ACID, OLEANOLIC ACETATE AND "MIXED LIPPIA ACIDS" ON MITOCHONDRIA ISOLATED FROM BILIARY FISTULA RABBITS RECEIVING THE TRITERPENES

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Abstract—The effect was studied of Icterogenin, crude Rehmannic acid, oleanolic acetate and "Mixed Lippia acids" on respiration, mitochondrial nitrogen content, oxidative phosphorylation and respiratory control in mitochondria isolated from rabbits receiving the triterpenes.

All triterpenes were found to increase the respiration rate with succinate as substrate, the increase being more marked with Icterogenin and "Mixed Lippia acids". Respiration with the NAD-linked enzymes was affected to a different degree. Icterogenin, "Mixed Lippia acids" and crude Rehmannic acid caused a slight ageing effect.

The mitochondrial nitrogen content was decreased, the oxidative phosphorylation was generally maintained, but the respiratory control was lowered.

The connection between oxidative phosphorylation and bile secretion is discussed and the possible connection betweeen chemical structure and the inhibition of bilirubin secretion.

A mechanism for this interference by the triterpenes is proposed.

IN A PREVIOUS paper[1] the triterpenes Icterogenin, Rehmannic acid and oleanolic acetate have been shown to affect oxidative phosphorylation by rat and rabbit liver mitochondria *in vitro*. There appears to be a correlation between the effect of these substances on oxidative phosphorylation and on biliary secretion.

The problem resolved itself into determining whether or not the *in vitro* findings indeed have relevance to the *in vivo* action of these substances. Such a connection would rest on the assumption that the compounds do not undergo structural changes *in vivo* that would invalidate comparison with their *in vitro* activity. Referring to the structural formulae of the triterpenes under consideration, especial interest attaches to the side chain at C 22, which has been found to be of importance for their effect on oxidative phosphorylation. Brown *et al.*[2] found in *in vivo* experiments that removal of this side chain from an active compound causes loss of potency in terms of activity which includes an effect on both bile flow and bilirubin content. Esterification of the C 24 hydroxyl group resulted in diminished potency. Chemical changes of this nature do not seem to occur *in vivo* under the conditions used here.

The question also arose whether the triterpenes exerted an effect on the mitochondria

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in vivo. By studying the release of enzymes into the blood stream after application of the materials and in choosing enzymes distributed in specific subcellular fractions according to Rees and Sinha[3] it was found that already between the first and second hour after application of the material the level of NADP-isocitric dehydrogenase, an enzyme known to be mainly in the liver cell sap,[4] had risen in the serum. This indicates that the triterpenes had been acting on the cellular membrane causing increased permeability with a resultant leakage of liver enzyme into the blood. The detection in the serum (3–5 hr after application of the material) of NAD-glutamic dehydrogenase, an enzyme known to be largely mitochondrial in localisation,[6] is a finding indicative of mitochondrial injury.[5] The triterpenes thus exert an effect upon the mitochondria *in vivo* as well as under *in vitro* conditions.

To determine whether mitochondrial oxidative phosphorylation was affected *in vivo*, and if other effects on the mitochondria could be detected, a study was undertaken of liver mitochondria isolated from biliary fistula animals receiving the triterpenes and sacrificed when the effect on biliary secretion was apparent.

MATERIALS AND METHODS

Male rabbits, weighing about 2 kg and fasted for 24 hr, were anaesthetised with ether and the biliary duct cannulated as described by Heikel *et al.*[7] and the cystic duct ligated. The material, suspended in 2% (w/v) Cellofas (medium viscosity, Imperial Chemical Industries), was introduced into the peritoneal cavity at the time of operation.[5] The animals were killed by cervical dislocation at such time when the effect upon biliary secretion had become marked (about 10 hr after operation and dosing).

Mitochondria were prepared as described in the preceding paper both from biliary fistula rabbits receiving the triterpenes and, as controls, from normal rabbits and biliary fistula rabbits not receiving the triterpenes. Respiration and oxidative phosphorylation experiments were performed using the same methods as before.[1]

Pure Icterogenin and oleanolic acetate were used in some experiments. The crude Rehmannic acid was examined chromatographically according to the method of Tschesche and Poppel[8] as modified by Anderson, de Kock and Enslin.[9] The preparation was found to contain a small amount of a substance, R_f 0·10, giving a blue colour with the Carr–Price reagent used for detection of triterpenes. Comparison of a variety of triterpenes by chromatography and application of this test revealed a correlation between chemical structure and the colour produced by the Carr–Price reagent;[5] a C 3 keto group is responsible for an orange colour whereas a hydroxyl group at the same position results in a rich colour change terminating in a blue colour. Components with a substituted hydroxyl group also showed this blue colour. As the unknown substance accompanying Rehmannic acid gives a terminal blue colour with the Carr–Price reagent and is negative to the Zimmermann reagent for ketone groups (negative Zimmermann being obtained only with 3 keto-triterpenoids having a methylene ketone structure[10]), the presence of a hydroxyl or substituted hydroxyl group seemed likely. Chromic acid oxidation of the material followed by chromatography gave evidence for a ketone group. Alkaline hydrolysis followed by chromatography altered the R_f value from 0·10 to 0·19 which is closely similar to that of oleanolic acid.[5]

These reactions suggest that material present in small amounts in the crude Rehmannic acid is 22 β-angeloyloxyoleanolic acid. This triterpene has indeed been isolated

from the root bark of Lippia Rehmanni.[9] Alkaline hydrolysis of this highly active substance gives 22 β-hydroxyoleanolic acid. Alkaline hydrolysis of the preparation "Mixed Lippia acids" gave rise to another compound, R_f 0·40–0·45, which also gave a blue colour with Carr–Price reagent. It seems likely, although experimental evidence is not presented, that this compound is formed from the triterpene acid 22 β-angeloyl-oxy-24-hydroxyoleanolic acid which has also been found in the root bark of Lippia Rehmanni.[9]

The preparation "Mixed Lippia acids" which we have used would thus appear to have contained 65% Icterogenin, 33% Rehmannic acid and small amounts of 22 β-angeloyloxyoleanolic acid and 22 β-angeloyloxy-24-hydroxyoleanolic acid.[2]

RESULTS

Respiration in a medium without added phosphate acceptor

Administration of Icterogenin and "Mixed Lippia acids" caused an increase in mitochondrial respiration rate using succinate as substrate; the increase was less pronounced in experiments with crude Rehmannic acid and oleanolic acetate. NAD-linked enzymes were affected to different degrees but a more or less pronounced increase in respiration rate was usually also found (Table 1).

TABLE 1. QO_2 VALUES WITH DIFFERENT SUBSTRATES OF MITOCHONDRIA FROM LIVERS OF RABBITS RECEIVING THE VARIOUS TRITERPENES

Exp. no.	QO_2 pyr.	QO_2 mal.	QO_2 glut.	QO_2 succ.	QO_2 oct.
		Controls (mean value ± S.E.M.)			
	181 ± 5·8	134 ± 3·5	153 ± 1·5	324 ± 7·6	263 ± 5·9
		"Mixed Lippia acids"			
25	231	141	210	525	346
28	258	175	239	540	358
27	198	144	201	559	317
30	251	158	239	708	354
29	535	196	268	586	210
		Icterogenin			
35	96	95	126	709	188
54	240	185	238	386	426
		Crude Rehmannic acid			
37	218	152	214	413	338
41	247	165	227	455	344
		oleanolic acetate			
42	62	49	80	617	52
43	267	163	237	439	357

Respiration in a medium without added phosphate acceptor.
pyr. = pyruvate; mal. = malate; glut. = glutamate; succ. = succinate; oct. = octanoate.

Respiration was also measured after ageing suspensions in 0·25 M sucrose at 0°, of mitochondria prepared from livers of biliary fistula rabbits receiving the triterpenes, and from control rabbits. In these experiments, malate and glutamate were used as substrates. The time of ageing was about 7 hr except for experiment No. 54 where 5 hr was measured from the death of the animals until the use of the preparation. Table 2 records the results of these experiments.

In mitochondria from triterpene treated animals the respiration decreased with ageing in all experiments except one, in contrast to the normal animals where the

respiration increased upon ageing. The rate of oxygen uptake was linear in all these experiments during the time of measurement.

Effect on mitochondrial nitrogen

Calculation of the nitrogen content of the mitochondria equivalent to 1 g wet wt. liver of the fraction studied revealed great differences in the nitrogen content in the

TABLE 2. EFFECT OF AGEING ON QO_2 VALUES WITH MITOCHONDRIA FROM LIVERS OF RABBITS RECEIVING THE VARIOUS TRITERPENES

Exp. no.	Substrate glutamate QO_2	aged QO_2	Substrate malate QO_2	aged QO_2
		Controls		
48	153	208 (136)	136	182 (134)
51	153	212 (139)	123	176 (143)
		"Mixed Lippia acids"		
29	268	232 (87)	196	177 (90)
30	239	221 (92)	158	136 (86)
		Icterogenin		
35	126	66 (52)	95	33 (35)
54	238	202 (85)	185	147 (79)
		Crude Rehmannic acid		
37	214	205 (96)	152	139 (91)
41	227	236 (104)	165	172 (104)

Respiration in a medium without added phosphate acceptor. The values in brackets indicate percentages of original QO_2 values.

TABLE 3. NITROGEN CONTENT OF THE MITOCHONDRIAL FRACTION FROM LIVERS OF RABBITS RECEIVING THE TRITERPENES

Exp. no.		mg N
	Controls (mean value ± S.E.M.)	3·31 ± 0·10
24	"Mixed Lippia acids"	1·52
25		3·02
27		2·24
28		1·29
29		1·97
30		1·43
	Icterogenin	
35		2·85
54		1·57
	Crude Rehmannic acid	
37		1·82
41		4·05
	oleanolic acetate	
42		1·32
43		2·28

The values are expressed as mg N equivalent to 1 g wet wt. liver.

various experiments (Table 3). In spite of the variation in consistency of the livers, the nitrogen content of the fraction in the control animals was in the range 3–4 mg/1 g wet wt. liver. In many experiments with the triterpenes, only about 50 per cent of this value was found. The experiments recorded in the previous paper indicate with much

certainty that the mitochondria become more or less damaged by the drugs. Damaged mitochondria are more susceptible to disruption during preparation than normal ones and the reduction in mitochondrial yield may be due to this fact. However, a true reduction in mitochondria during the experiment with the triterpenes cannot be excluded.

Effect on oxidative phosphorylation

Measurement of oxidative phosphorylation by mitochondria from livers of biliary fistula rabbits receiving the triterpenes revealed that the mitochondria had retained their capacity to carry out oxidative phosphorylation. No significant differences from normal were seen in experiments with glutamate or succinate as substrates (Table 4). No phosphorylation measurements were done with other substrates. In those experiments where a lowered P/O ratio was obtained it was noted that the bile flow had nearly ceased, indicating, presumably, a very severe effect of the material. The rate of oxygen uptake in a medium with added phosphate acceptor was not linear declining slightly during the second period of measurement.

TABLE 4. OXIDATIVE PHOSPHORYLATION WITH MITOCHONDRIA FROM LIVERS OF RABBITS RECEIVING THE TRITERPENES

Exp. no.	P/O glutamate	P/O succinate
	Controls (mean value ± S.E.M.)	
	2·13 ± 0·10	1·32 ± 0·26
	"Mixed Lippia acids"	
25	2·09	1·24
28	2·17	1·25
27	1·98	0·98
30	1·99	1·13
29	1·93	1·01
	Icterogenin	
35	1·15	0·21
54	2·08	1·13
	Crude Rehmannic acid	
37	2·10	1·08
41	2·01	1·06
	oleanolic acetate	
42	1·98	0·47
43	1·89	1·34

Respiratory Control

Respiratory control is regarded as a reliable index for the integrity of the mitochondria. The rate of respiration in the absence of a phosphate acceptor is measured, the latter is then introduced and the increase in rate is considered as the degree of the control. The respiratory control in mitochondria from livers of rabbits with biliary fistulae and receiving the triterpenes is recorded in Table 5. The respiratory control, generally between 3 and 4 in control animals, was lowered in most experiments.

DISCUSSION

In a previous paper[1] it has been shown that Icterogenin, Rehmannic acid and oleanolic acetate added to mitochondrial preparations affect the oxidative phosphorylation;

Icterogenin and Rehmannic acid were found to uncouple it. All three substances increased mitochondrial membrane permeability and Icterogenin exerted a swelling effect.

TABLE 5. RESPIRATORY CONTROL IN MITOCHONDRIA FROM LIVERS OF RABBITS RECEIVING THE VARIOUS TRITERPENES

Exp. no.	Respiratory control	
	Glutamate	Succinate
	Controls (mean value ± S.E.M.)	
	3·34 ± 0·17	3·39 ± 0·26
	"Mixed Lippia acids"	
25	2·07	2·30
28	2·72	2·51
27	2·38	1·97
30	1·83	1·49
29	1·86	1·72
	Icterogenin	
35	1·9	0·83
54	3·04	3·02
	Crude Rehmannic acid	
37	1·91	1·99
41	2·13	2·34
	oleanolic acetate	
42	1·26	0·83
43	1·71	2·22

The values are calculated for a 10 min period.

Isolated mitochondria from livers of animals to which the triterpenes had been administered showed in the present study changes from normal, indicated usually by increased respiration, lowered respiratory control and decreased mitochondrial nitrogen content in the fraction studied.

Thus it has been shown that the triterpenes act on the mitochondria *in vivo*. That the effect of the triterpenes was directed to the liver mitochondria is probably a consequence of the manner of application of these substances. The liver is almost certainly the first organ reached by a major part of the materials but an action on other organs cannot be excluded.

It may also be questioned whether the effect of the triterpenes is limited to the mitochondria or whether they possibly exert an effect also on the membrane of the whole cell. By studying the release of enzymes into the blood stream after application of the materials, evidence was obtained for an action of the triterpenes on the plasma membrane.[5] However, this effect on the plasma membrane showed no correlation with the effect on biliary secretion. One is therefore tempted to correlate the effect of the triterpenes on bile flow with their effect on oxidative phosphorylation. In view of previous failure to find a close correlation between total liver ATP and bile flow[11] it appears possible that, if ATP is involved in bile secretion, then it may be contained within a relatively small intracellular compartment. Preliminary evidence supports such an hypothesis (T. F. Slater, personal communication). The effect of the triterpenes on oxidative phosphorylation appears to be largely, although not entirely, dependent upon the presence of an angeloyloxy side chain attached to C 22 of the molecule.

Considering now the effect on bilirubin secretion, it has been shown that 22 β-angeloyloxyoleanolic acid is the most potent icterogenically active compound.[2] The strong effect exerted by this compound on bile flow can be appreciated, on the basis of structural similarities, by assuming an effect on oxidative phosphorylation similar to that of Rehmannic acid. The α-axial epimer of 22 β-angeloyloxyoleanolic acid exerts an effect only on the volume of bile secreted, and this effect is of the same order as that produced by the β-equatorial compound.[2]

Unfortunately the α-axial epimer was not available to test whether or not it had any effect on oxidative phosphorylation. Nevertheless one is tempted to connect the β-C 3 hydroxyl group with the decrease in bilirubin secretion. A possible conjugation of glucuronic acid with the β hydroxyl group in the triterpenes might account for the decrease in bilirubin elimination should competition for glucuronidation assume importance when the available energy for secretion was low. The strong effect of 22 β-angeloyloxyoleanolic acid could be explained by assuming such a mechanism, if its effect on oxidative phosphorylation were comparable to that of Rehmannic acid. An interference with the actual secretion of glucuronides is another possibility, but further data are necessary before these questions can be solved.

As 22 β-angeloyloxyoleanolic acid resembles Icterogenin in causing prompt hemolysis of erythrocytes, the following mechanism for the action of icterogenically active triterpenes on the biliary secretion is tentatively proposed. The substances affect the liver cell, causing an increased permeability of the plasma membrane which, in turn, results in release of cell sap enzymes into the bloodstream. In the cell they exert an effect on the mitochondrial membrane causing hydrolysis of an energy-rich phosphate intermediate with resultant uncoupling and activation of a latent ATPase which will decrease the ATP derived from other sources. Due to loss of Mg^{2+}, the activated ATPase will first become limited in its activity, then more severely inhibited. Histochemical evidence for decreased ATPase at the bile canaliculi following the administration of icterogenin has been provided by Goldfischer *et al.*[12] Thus the triterpenes decrease the ATP and limit ATPase activity, both of which have frequently been regarded as implicated in active transport.

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