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STUDIES OF FREE AND PROTEIN-BOUND β-FLAVASPIDIC ACID-*N*-METHYLGLUCAMINATE IN ISOLATED RAT HEPATOCYTES AND MITOCHONDRIA*

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Abstract—β-Flavaspidic acid-*N*-methylglucaminate (FLAV), the water-soluble derivative of flavaspidic acid, was absorbed from medium by isolated hepatocytes at a concentration-dependent rate. At a medium concentration of 5 mM, FLAV was absorbed at a steady state rate of 1.2 nmoles/mg of protein/min. Oxygen consumption by the cells was increased erratically by 0.18 nmole/mg of protein/min, a quantity not considered sufficient to support microsomal oxidation of the compound. When added to mitochondrial suspensions, FLAV at concentrations ranging from 80 nM to 8 μM uncoupled oxidative phosphorylation and caused a linear stimulation of ADP-independent respiration. FLAV binds readily to bovine serum albumin (BSA) and to the fatty acid-binding protein (FABP) in the cytosol. When bound to these proteins in molar ratios of 0.66 and 3.3, respectively, or to a partially purified cytosolic FABP protein (12.7 nmoles/μg of protein), FLAV failed to uncouple oxidative phosphorylation. These data suggest that, at a medium concentration of 5 mM, FLAV does not alter the mitochondrial oxidative activity of isolated hepatocytes. Thus, results from studies in isolated hepatocytes utilizing FLAV reflect the displacement of fatty acids from FABP and are not due to a secondary metabolic effect of the compound at the level of oxidative phosphorylation.

β-Flavaspidic acid-*N*-methylglucaminate (FLAV), a water-soluble phloroglucinol derivative extracted from male fern (*Dryopteris filix-mas*) [1–4], competes with free fatty acids and their CoA thioesters for binding to a low molecular weight protein (12,000 mol. wt) present in the cytosol of hepatic, jejunal, cardiac and adipose tissues [5, 6]. This selective affinity of FLAV for the fatty acid-binding protein (FABP) has been employed in a number of investigations. When added to liver perfusate [6], isolated hepatocytes [7] and everted jejunal sacs [8], FLAV markedly decreased the flux of fatty acids toward esterification. In perfused liver and hepatocytes, the decrease in esterification was reflected in a decreased uptake of fatty acids; in everted sacs, the tissue level of free fatty acids increased as esterification decreased.

Perfusion of the liver with FLAV did not stimulate oleate-supported oxygen consumption [6]; similarly, incubation of isolated hepatocytes with FLAV did not increase total oleate-supported CO_2 and ketone body production [7]. However, FLAV added to everted jejunal sacs caused a 60 per cent decrease in oleate-supported CO_2 formation [8]. Although FLAV added to this system had no effect on acetate oxidation, glucose oxidation was stimulated by 70 per cent, which was attributed to uncoupling of oxidative phosphorylation. Ockner and Manning [8] also cited unpublished results of Burnett and Ockner which were consistent with a concentration-dependent uncoupling effect of FLAV. In isolated hepatocytes they reported that the compound increased oxygen consumption and conversion of oleate, acetate and glucose to CO_2. Earlier work by Runeberg [9] demonstrated that flavaspidic acid added to mitochondrial suspensions in concentrations of 8–64 μM uncoupled oxidative phosphorylation. Paradoxically, oxygen consumption also was decreased when the system contained a terminal phosphate acceptor. In the absence of the terminal phosphate acceptor, 32 μM but neither 16 μM nor 64 μM FLAV stimulated oxygen consumption to the level observed in the presence of either the terminal phosphate acceptor or 0.1 mM 2,4-dinitrophenol.

Because of these observations it seemed necessary to re-examine certain effects of FLAV on isolated rat liver mitochondria and hepatocytes. The principal action that the compound exerts via the displacement of fatty acids from FABP could be then better evaluated as a control mechanism in lipid metabolism.

MATERIALS AND METHODS

Livers from 250-g male rats fed *ad lib.* were washed, minced and finely homogenized in chilled medium consisting of 224 mM mannitol, 75 mM sucrose and 0.1 mM EDTA using a Potter–Elvehjem type homogenizer. The mitochondria were isolated

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from a 10% homogenate by differential centrifugation according to the method of Schneider [10] as modified by Johnson and Lardy [11]. The heavy mitochondrial pellet was suspended in the above medium at a protein concentration of 17 mg/ml. The cytosol fraction was prepared from a 33% homogenate which was centrifuged directly at 105,000 *g* for 2 hr in a Beckman model L3-50 ultracentrifuge. To obtain the partially purified FABP, 2.0 ml cytosol was placed on a 2.8 × 74 cm Sephadex G-100 column and the proteins were eluted with 10 mM phosphate buffer, pH 7.4, at a flow rate of 32 ml/hr. The protein concentration of the eluate was monitored at 280 nm. Aliquots of 0.5 ml from all 5-ml fractions exhibiting absorbance at 280 nm were pooled and lyophilized, and called the cytosolic protein fraction used in subsequent experiments. The FABP was identified in cytosolic protein fractions 50–55 based on their high affinity for both oleic acid and sulfobromophthalein. Fraction numbers 52 and 53 were pooled separately and lyophilized to provide the FABP.

Hepatocytes were isolated from livers after collagenase perfusion according to the method of Berry and Friend [12] as modified by Zahlten *et al.* [13]. The hepatocytes suspended in a calcium-free Krebs–Ringer bicarbonate buffer, pH 7.4, at protein concentrations of 15 or 86 mg/ml were held at ice temperature under an atmosphere of 95% O_2–5% CO_2.

To study the uptake of FLAV by hepatocytes, 1-ml aliquots of the cell suspension (15 mg protein) were added to 2.5 × 5 cm glass vials containing 1 ml of calcium-free Krebs–Ringer bicarbonate buffer, pH 7.4, 3% bovine serum albumin (BSA), 3 μmoles Na oleate and FLAV. After incubation at 37° in a Dubnoff incubator (100 c.p.m.) for 30 min in the presence of 0–15 mM FLAV or in the presence of 5 mM FLAV for 0–60 min, the cell suspensions were diluted with 2 vol. of 150 mM NaCl at ice temperature. After centrifugation, the concentration of FLAV remaining in the cell-free diluted medium was determined spectrophotometrically with an Aminco DW-2 split-beam spectrophotometer. The ultraviolet spectrum of FLAV in water (Fig. 1) was identical to that published for an ethanolic solution of flavaspidic acid [3]. The molar absorption coefficient at 298 nm was $1.67 \times 10^4\ M^{-1}\ cm^{-1}$. The absorption spectrum of 0.05 mM FLAV in 1.5% aqueous albumin is also shown in Fig. 1. The absorption maximum was shifted from 298 to 310 nm. The ϵ_{310} was $1.47 \times 10^4\ M^{-1}\ cm^{-1}$. Absorbance at 310 nm of FLAV in aqueous albumin was linear up to 0.1 mM (Fig. 2).

Oxygen consumption, P–O ratios and respiratory control ratios were determined by the method of Estabrook [14] using a Gilson Oxygraph equipped with a Clark oxygen electrode. A 0.1-ml aliquot of mitochondrial suspension (1.7 mg protein) was added to 1 ml buffer consisting of 20 mM KCl, 225 mM sucrose, 10 mM KH_2PO_4, 5 mM $MgCl_2$ and 20 mM triethanolamine–HCl, pH 7.4. State 4 respiration was determined after addition of 4.5 μmoles succinate and state 3 respiration after the addition of 0.58 μmole ADP. The initial oxygen concentration of the buffer at 24° was calculated to be 0.26 mM. The concentration of FLAV when present was in the range 80 nM–8 μM. Proteins, known to bind FLAV, were added to the electrode chamber in the following concentration ranges: BSA, 0.25 to 1.0 mg/ml; cytosolic protein, 3.1 to 6.3 μg/ml; and FABP, 0.15 to 0.64 μg/ml. FLAV was mixed with the latter two proteins before being introduced into the chamber.

Oxygen consumption by the hepatocytes was also determined using the Clark oxygen electrode as described by Primack and Buchanan [15]. A 0.1-ml aliquot of the cell suspension (8.6 mg protein) was added to 1.2 ml buffer equilibrated with room air. The buffer consisted of 135 mM NaCl, 5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 10 mM

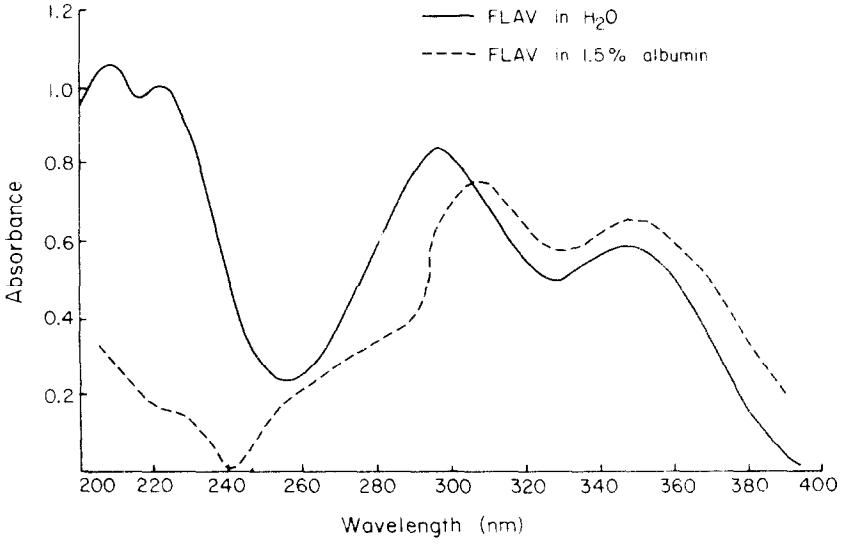

Fig. 1. Ultraviolet absorption spectra of flavaspidic acid-*N*-methylglucaminate dissolved in water (———) and in 1.5% albumin (- - -). FLAV at a concentration of 0.05 mM in the sample cell was read against either a water or 1.5% albumin reference blank.

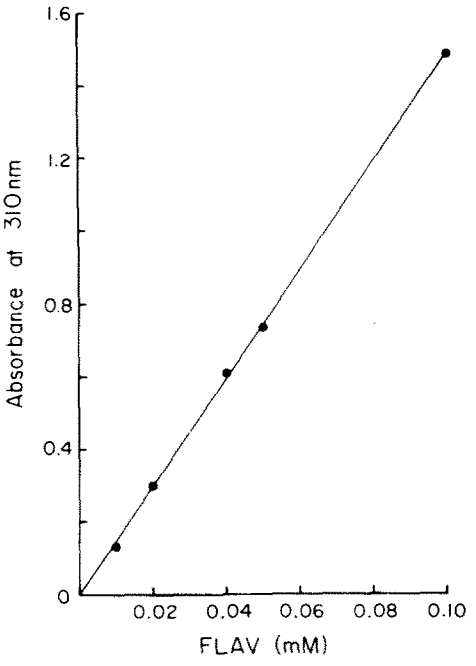

Fig. 2. Linear absorbance of FLAV in 1.5% albumin at 310 nm.

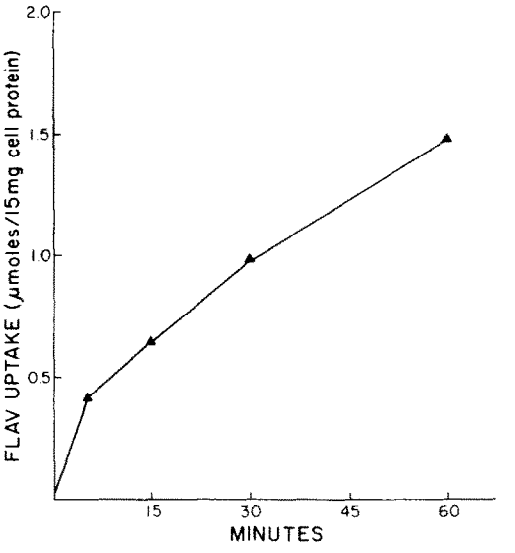

Fig. 3. Rate of FLAV uptake by isolated hepatocytes. Hepatocytes (15 mg of cell protein) were incubated in 2.0 ml of a Ca^{2+}-free bicarbonate buffer containing 1.5% albumin, 3.0 nmoles Na oleate and 10 μmoles FLAV. The uptake of FLAV was determined by measuring the disappearance of FLAV from the medium. The data represent an average of two experiments.

glucose in 5 mM Tris–HCl, pH 7.4. FLAV, when present, was at a concentration of 5 mM.

Protein was determined according to Biuret reaction [16].

Rats were obtained from Holtzman Farms, Madison, WI. Collagenase (CLS II) was purchased from Worthington Biochemical Corp., Freehold, NJ. β-Flavaspidic acid-*N*-methylglucaminate was generously provided by Dr. Esa Aho, Turku, Finland. All other chemicals were of the highest reagent grade commercially available.

RESULTS

The uptake of FLAV by hepatocytes was time- and concentration-dependent. Hepatocytes were incubated in medium containing 5 mM FLAV, and the rate of uptake of the compound was calculated at various time intervals from the data in Fig. 3. During the first 5 min there was an initial rate of uptake of 5.4 nmoles FLAV/mg of protein/min. The rate then decreased to 1.5 nmoles FLAV/mg of protein/min, and, during the final 30 min of incubation, the average rate of uptake was 1.2 nmoles FLAV/mg of protein/min. The average rates of uptake during a 30-min incubation were calculated to be 0.005 nmole, 0.5 nmole, 2.2 nmoles, 3.64 nmoles and 4.9 nmoles FLAV/mg of protein/min when the hepatocytes were incubated in medium containing 0.1, 1.0, 5.0, 10.0 and 15.0 mM concentrations of FLAV respectively (Fig. 4).

We have previously reported [7] that mitochondrial oxidative metabolism of oleate is indirectly influenced by FLAV whereas microsomal esterification activity is directly inhibited at concentrations of FLAV ranging up to 10 mM. This conclusion was substantiated by the present experiments measuring the oxygen uptake by isolated hepatocytes in the Clark oxygen electrode chamber. Hepatocytes consumed oxygen at the rate of 9.12 ± 0.01 μl O_2/mg of protein/hr in the absence of FLAV and 9.34 ± 1.01 μl O_2/mg of protein/hr in the presence of 5 mM FLAV.

Mitochondria utilized in these studies had respiratory control values of 7.0 (Fig. 5a) with a succinate-supported ADP/O ratio of 1.71 ± 0.14 (Table 1). The ADP-independent oxygen uptake by mitochondria was increased from a control rate of 9

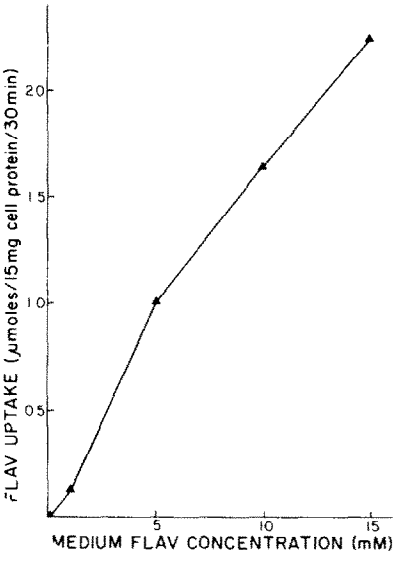

Fig. 4. Effect of concentration of FLAV in the medium on its uptake by isolated hepatocytes. Hepatocytes were incubated as described in Fig. 3. FLAV uptake equals the initial minus the concentration recovered at the end of the 30-min incubation period. The values are an average of two experiments.

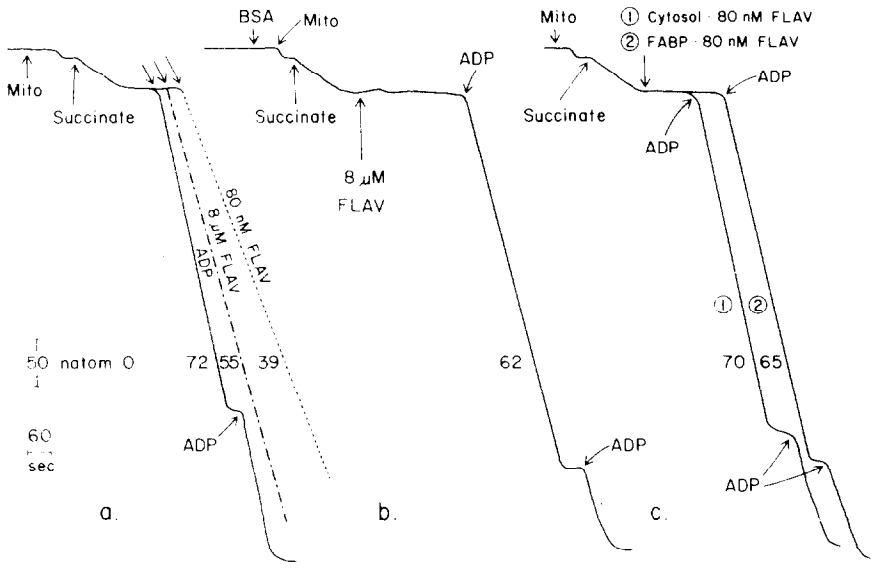

Fig. 5. Effect of FLAV, BSA, cytosolic protein and FABP on mitochondrial respiration. The medium contained in 1.1 ml: 20 mM KCl, 225 mM sucrose, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 20 mM triethanolamine-HCl, pH 7.4, 24° and 1.7 mg of mitochondrial protein. Other additions were made as shown. Numbers on the tracing indicate the rates of oxygen consumption in natoms/min/mg of protein.

natoms O/mg of protein/min to rates of 39 and 55 natoms O/mg of protein/min by the addition of 80 nM and 8 μM FLAV, respectively, to the electrode chamber (Table 1 and Fig. 5a). The effectiveness of FLAV as a stimulator of ADP-independent respiration was attenuated by the sequential additions of BSA and FLAV. Increasing the BSA concentration from 0.25 to 1.0 mg/ml (Table 1) decreased the respiratory response to 8 μM FLAV, and at the highest level of BSA, oxidation was coupled to phosphorylation (Table 1 and Fig. 5b), resulting in a succinate-supported ADP–O ratio of 1.47. Subsequently, microgram quantities of the cytosolic protein and FABP were mixed with FLAV prior to their introduction into the electrode chamber. The stimulatory response of ADP-independent respiration by FLAV was decreased linearly as the ratio of FLAV to the binding protein was decreased (Table 1). The introduction of FLAV with cytosolic protein (12.7 nmoles/μg of protein) into the electrode chamber did not stimulate ADP-independent respiration. FLAV : FABP in a molar ratio of 3.3 also failed to stimulate respiration (Fig. 5c). At higher ratios, partial stimulation of ADP-independent respiration was observed (Table 1). Addition of FLAV complexed to proteins in the ratios noted above failed to uncouple oxidative phosphorylation (Fig. 5c) as succinate-supported ADP-O ratios were observed to be within the control range. However, ADP-independent respiration in the presence of FLAV complexed to protein was slightly inhibited (Table 1 and Fig. 5b and c).

DISCUSSION

FLAV has been widely used as an adjunct in the study of fatty acid metabolism. Any effect of FLAV on cell metabolism other than that mediated through

Table 1. Effects of FLAV and FABP on the rat liver mitochondrial respiration

FLAV concn	Protein concn	$\bar{v}$*	Oxygen consumption (natoms O/min/mg)				
			State 4	Uncoupled†	State 3	R.C.	ADP–O
0	0		9		72	8.0	1.71
80 nM	0			39			
8 μM	0			55			
8 μM	BSA (1 mg)	0.66	11		62	5.6	1.47
	BSA (0.5 mg)	1.32		25			
	BSA (0.25 mg)	2.64		42			
80 nM	Cytosol (6.3 μg)		9		70	7.8	1.66
	Cytosol (3.1 μg)			24			
80 nM	FABP (0.64 μg)	3.30	9		65	7.2	1.54
	FABP (0.40 μg)	5.28		25			
	FABP (0.20 μg)	10.56		35			
	FABP (0.15 μg)	14.00		39			

* Molar ratio of FLAV to the added protein.
† Stimulation of respiration after FLAV; no ADP present.

its competitive binding to FABP could lead to erroneous interpretations of experimental results.

In general, these studies support observations showing that FLAV is readily taken up from surrounding medium by intact liver [17, 18], perfused liver [6] and isolated hepatocytes [7] in a concentration-dependent fashion. The results obtained from experiments with isolated mitochondria are consistent with Runeberg's report [9] that the uncoupling effect of mitochondrial respiration in the Warburg apparatus was initiated by a concentration of FLAV between 8 and 16 μM. Additional insight into the mode of action of FLAV is provided by our observation that this uncoupling effect of FLAV is attenuated by the binding of FLAV to specific proteins, and at specific molar ratios (0.66 $\bar{v}$ to BSA, 3.3 $\bar{v}$ to partially purified FABP and 10.0 $\bar{v}$ to endogenous FABP on the assumption that only FABP in the cytosol protein binds FLAV) the uncoupling effect of FLAV is abolished. Under these conditions, FLAV may slightly inhibit state 3 respiration but state 4 respiration, respiratory control ratios and succinate-supported ADP–O ratios fall within the range of normal values.

In agreement with our earlier findings [7], we were unable to demonstrate that FLAV at a medium concentration up to 5 mM uncoupled oxidative phosphorylation in isolated hepatocytes. These hepatocytes exhibited only a 2.7 per cent higher rate of oxygen consumption than that of control hepatocytes. Endogenous FABP must, therefore, provide adequate capacity to bind the intracellular FLAV. By extrapolation, our data suggest that 15 mg of hepatocyte protein provides 10 nmoles of intracellular FABP, which represents 2 per cent of the cytosolic protein [19, 20], an amount sufficient to bind 100 nmoles FLAV. The initial rate of absorption of FLAV (Fig. 1) probably reflects the rate at which the compound complexes with, and saturates, the endogenous FABP. Presumably, the quantity of FLAV present in the hepatocyte bound to FABP increased slightly, if at all, during the remainder of the incubation periods. The absorption of hydrophobic molecules, e.g. free fatty acids, from incubation medium follows a similar pattern in that there is an initial rapid uptake, dictated possibly by their binding to FABP, followed by a slower rate of uptake which is proportional to the entry of fatty acids into the esterified lipid fraction [21].

Although the relationship of FLAV to the FABP has been well characterized, its metabolism in tissues subsequent to binding is unclear. Neither the water-soluble derivative FLAV nor flavaspidic acid accumulates in perfused tissues [17, 18], but the route of excretion for these compounds has not been identified [18, 22]. Treatment of human tapeworm infestation with an extract of male fern which contains phloroglucinol derivatives regularly leads to transient hyperbilirubinemia and increased retention of sulfobromophthalein, but does not elicit general hepatocellular damage [17]. The present studies carried out with isolated hepatocytes are able to clarify some of the effects of FLAV observed *in vitro*, but they do not permit any further understanding of its metabolism *in vivo*.

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