Effect of 3-Hydroxyanthranilic Acid on the Mitochondrial Respiratory System

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Studies on the metabolites of tryptophan in urine, in various neoplastic diseases, have shown an unusual excretion pattern, some of these compounds being eliminated in greater amounts than in normal human beings (Boyland & Williams, 1956*a, b*; Musajo, Benassi & Parpajola, 1955; Quagliariello, Tancredi, Fedele & Saccone, 1961; Tompsett, 1959; Ivanova & Ramonova-Tskhovrebova, 1960; Price & Brown, 1962). Particularly in bladder cancer there appeared to be an excretion of considerable quantities of 3-hydroxyanthranilic acid and 3-hydroxykynurenine. The significance of the accumulation in the body of these biological *o*-aminophenols in relation to neoplastic disease is still not clear. However, a number of *o*-aminophenols are known to induce bladder cancer (Boyland, 1958), and it has been shown that 3-hydroxyanthranilic acid and 3-hydroxykynurenine can induce experimental cancer of the bladder (Allen, Boyland, Dukes, Horning & Watson, 1957), and 3-hydroxyanthranilic acid some forms of leukaemia (Ehrhart & Georgii, 1959). Further, Dunning, Curtis & Mann (1950) reported that bladder cancer caused by 2-acetamidofluorene arises in practically all cases in rats when the diet is supplemented with tryptophan.

We decided to investigate the effects of these tryptophan metabolites on the main cellular processes, and first studied the effect of 3-hydroxyanthranilic acid on mitochondrial oxidative phosphorylation. It is generally known that substituted phenols (thyroxine, dinitrophenols etc.) act on mitochondrial processes.

Quagliariello, Saccone, Rinaldi & Alioto (1959) have shown an antagonism between 3-hydroxyanthranilic acid and vitamin K. The structural configuration of 3-hydroxyanthranilic acid is such that it may account for its antagonism towards biological naphthaquinones and benzoquinones (see also Mentzer, 1948), which may play a part in the oxidative phosphorylation.

3-Hydroxyanthranilic acid apparently uncoupled oxidative phosphorylation and inhibited the oxidation of α -oxoglutarate by rat-liver and rat-heart mitochondria (Quagliariello, Papa & Saccone, 1962*a, b*). It also activated glycolysis in a fraction of rat liver (Papa, Budillon & Quagliariello, 1962*b*)

and increased the incorporation of labelled amino acids into the proteins of rat-liver preparations (Barnabei, Papa & Quagliariello, 1963). The present paper is concerned with a detailed study of the effect of 3-hydroxyanthranilic acid on the oxidation of α -oxoglutarate and on other NAD-dependent mitochondrial oxidations. The effect of 3-hydroxyanthranilic acid on adenosine triphosphatase and on the structural state of mitochondria is also described.

EXPERIMENTAL

Materials. The following commercial products were used: α -oxoglutaric acid, sodium succinate hexahydrate, sodium pyruvate, sodium fumarate, NAD (free acid), ATP (disodium salt, trihydrate), ADP (trisodium salt, monohydrate), tris and triethanolamine hydrochloride (all from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany); sodium DL- β -hydroxybutyrate (from Mann Research Laboratories, New York, N.Y., U.S.A.); L-malic acid and yeast hexokinase (type III) (from Sigma Chemical Co., St Louis, Mo., U.S.A.); sodium malonate (from K. and K. Laboratories Inc., New York, N.Y., U.S.A.); 2,4-dinitrophenol (from E. Gurr Ltd., London); 3-hydroxyanthranilic acid, 3-hydroxy-DL-kynurenine, xanthurenic acid, kynurenic acid and DL-isocitrate (trisodium salt, monohydrate) (all from L. Light and Co. Ltd., Colnbrook, Bucks.); DL-tryptophan and DL-kynurenine (both from Hoffmann-La Roche, Basle, Switzerland); anthranilic acid (from C. Erba, Milan, Italy); crystalline bacterial proteinase (Nagarse) (from Teikoku Chemical Industry Co. Ltd., Osaka, Japan), and L-glutamic acid, EDTA and other common chemicals (from E. Merck A.-G., Darmstadt, Germany).

Preparation of rat-liver mitochondria. Male Wistar albino rats, weighing 250–300 g. and kept without food for 12 hr., were used in all experiments. The animals were decapitated in the cold room. The liver was immediately removed, washed with an ice-cooled medium containing sucrose (0.25M) and EDTA (1 mM) in 1 mM-tris buffer, pH 7.4, and forced through a fine steel mesh. Approx. 7–8 g. of liver was homogenized in 10 vol. of the sucrose (0.25M)–EDTA (1 mM) medium in 1 mM-tris buffer, pH 7.4, in a Potter-Elvehjem homogenizer with a Teflon pestle working at 1000 rev./min. for 1 min. Mitochondria were isolated by a modification of the procedure of Schneider & Hogeboom (1950). The homogenate was centrifuged at 800*g* for 10 min. in a Lourdes Super-Speed centrifuge. The supernatant was centrifuged at 6000*g* for 10 min.; the sediment, resuspended in the sucrose (0.25M)–EDTA (1 mM) medium in 1 mM-tris

buffer, pH 7.4, was centrifuged at 800g for 5 min. The slight sediment was discarded and the mitochondrial fraction was collected at 6000g for 10 min. The pellet thus obtained was washed once at 12 000g. The fluffy layer was carefully removed and the compact mitochondrial sediment was suspended in 0.25 M-sucrose, at a concentration equivalent to 1 g. of liver/ml.

Preparation of rat-heart mitochondria. The heart, washed with a medium containing sucrose (0.3M) and EDTA (1 mM) in 10 mM-triethanolamine hydrochloride buffer, pH 7.2, was cut into small pieces and suspended for 10 min. in the sucrose (0.3M)-EDTA (1 mM) medium in 10 mM-triethanolamine hydrochloride buffer, pH 7.2, in the presence of 5 mg. of bacterial proteinase. The suspension was homogenized for 1–2 min. at 1000 rev./min. and the homogenate was centrifuged at 800g for 10 min. The supernatant was centrifuged twice at 5000g for 10 min.; the mitochondrial fraction was washed once at 8000g, the fluffy layer was discarded and the compact sediment suspended in a medium containing KCl (0.13M) in 10 mM-triethanolamine hydrochloride buffer, pH 7.2, at a concentration equivalent to one heart/0.5 ml. All the operations were carried out at 0–4°. The purity and the integrity of both the mitochondrial fractions were tested by evaluating the P:O ratios, which were almost exactly equal to the theoretical values, and by observing the respiratory control with an oxygen electrode.

Mitochondrial respiration and oxidative phosphorylation. Mitochondrial respiration was measured both by the Warburg manometric technique and polarographically with a vibrating platinum electrode in a thermo-regulated cuvette (Oxygraph, model K; Gilson Medical Electronics, Middleton, Wis., U.S.A.). For the manometric method, mitochondria from 200 mg. of liver (3–4 mg. of protein) were incubated in 5 ml. Warburg vessels at 30° in a final volume of 1 ml. of one of the following media.

For experiments on oxidative phosphorylation, the medium contained (final concentrations): potassium phosphate buffer, pH 7.5 (20 mM), KCl (30 mM), glucose (30 mM), MgCl₂ (5 mM), KF (5 mM), tris buffer, pH 7.4 (20 mM), sucrose (60 mM), ATP (2 mM) and K.M. hexokinase [150 units (1 unit is defined as the amount of enzyme which catalyses the formation of 10 μ m-equiv. of acid/min. at 5° and pH 7.5 in the standard reaction mixture, as reported by McDonald, 1955), dissolved in 0.1 ml. of 0.15 M-substrate]. The hexokinase substrate solution was tipped into the main compartment from the side arm after 5 min. of equilibration. 3-Hydroxyanthranilic acid and other tryptophan metabolites, NAD and malonate were added directly to the main compartment at the final concentrations reported in the Tables and Figures. The disappearance of orthophosphate from the medium was determined by the method of Lowry & Lopez (1946).

Respiration in the presence of 2,4-dinitrophenol was measured in a medium containing (final concentrations): KCl (80 mM) [or KCl (50 mM) plus potassium phosphate buffer, pH 7.5 (20 mM)], 2,4-dinitrophenol (0.1 mM), MgCl₂ (5 mM), tris buffer, pH 7.4 (20 mM), sucrose (60 mM) and respiratory substrate (15 mM).

Rat-heart mitochondria were used for studies with the oxygen electrode. These mitochondria are particularly suitable for the study of the oxidation of a single substrate since their endogenous respiration falls to zero within 3–4 min. The mitochondria from 300 to 400 mg. of heart

were incubated at 25° in a final volume of 1 ml. of a medium containing KCl (0.13M) in 10 mM-triethanolamine hydrochloride buffer, pH 7.2.

The added amounts of substrates, cofactors and other substances are reported in the legends of the Figures.

Mitochondrial adenosine triphosphatase. The mitochondria from 100 mg. of rat liver (1.5–2 mg. of protein) were incubated at 30° in a final volume of 2 ml. containing (final concentrations): sucrose (150 mM), tris buffer, pH 7.4 (15 mM), ATP (5 mM), MgCl₂ (when present, 4 mM), 2,4-dinitrophenol (0.1 mM) and respiratory substrate (5 mM).

Mitochondrial swelling. Mitochondrial swelling was followed by observing the change in E_{292} , over a period of 30 min., of a suspension of mitochondria from 50 mg. of liver in 3 ml. of a medium containing KCl (0.125 M) in 20 mM tris buffer, pH 7.4. The initial E values ranged between 0.7 and 0.5 (Lehninger, 1959).

Determination of protein. Protein was determined by the method of Cleland & Slater (1953) and by a modified micro-Kjeldhal procedure.

RESULTS

Effect of 3-hydroxyanthranilic acid on respiration and phosphorylation of mitochondria oxidizing α -oxoglutarate or succinate. As reported by Quagliariello *et al.* (1962a, b), 3-hydroxyanthranilic acid added to tightly coupled mitochondria respiring with α -oxoglutarate as substrate, markedly inhibited the oxygen uptake at concentrations of 1–0.25 mM. At these concentrations oxidative phosphorylation was more strongly inhibited, thus giving apparent uncoupling.

In other experiments it was found that 30 mM-malonate decreased to about 50 % the oxygen uptake of mitochondria respiring with α -oxoglutarate as substrate. In these experiments the percentage inhibition by 3-hydroxyanthranilic acid of oxygen uptake was the same as that found in experiments performed in the absence of malonate; the percentage inhibition of phosphate uptake was doubled. The addition of NAD (1 mM) or of NAD plus manganous chloride (0.3–1 mM) did not reverse the inhibitory effects of 3-hydroxyanthranilic acid. Table 1 summarizes these results.

Further, the effect of 3-hydroxyanthranilic acid on the respiration of rat-heart mitochondria was studied. Fig. 1 (a) shows a polarographic record of oxygen uptake by heart mitochondria oxidizing α -oxoglutarate. The endogenous respiration of heart mitochondria dropped to zero after about 3 min. of incubation. On the addition of α -oxoglutarate (4 mM) in the presence of orthophosphate and malonate, respiration started again and was activated by the addition of ADP (0.4 mM); when the ADP had been utilized, respiration returned from the 'active state 3' (Chance & Williams, 1956) to the 'controlled state 4'. On the addition of 3-hydroxyanthranilic acid (0.5 mM) to the mitochondrial suspension at the beginning of the

Table 1. *Effect of 3-hydroxyanthranilic acid on respiration and oxidative phosphorylation of rat-liver mitochondria respiring with α -oxoglutarate as respiratory substrate*

The results refer to 1 ml. of mitochondrial suspension (3–4 mg. of protein). The composition of medium was that given in the Experimental section for experiments on oxidative phosphorylation. The incubation time was 15 min.

Expt. no.	Additions	Concn. of 3-hydroxy-anthranilic acid (mm)	Oxygen uptake (μ g.atoms)	Inhibition of respiration (%)	Phosphate esterified (μ moles)	P:O ratio	Inhibition of phosphorylation (%)
1	None	0.0	4.28	—	12.11	2.83	—
		1.0	0.90	79	1.76	1.95	31
		0.5	1.15	73	2.29	1.99	29
		0.25	2.14	50	4.88	2.28	19
		0.1	4.29	—	11.17	2.60	8
2	Malonate (30 mm)	0.0	2.14	—	7.90	3.69	—
		1.0	0.55	74	0.28	0.50	86
3	Malonate (30 mm) + NAD (1 mm)	0.0	2.28	—	8.53	3.74	—
		1.0	0.48	79	0.25	0.52	86
		0.5	0.72	68	1.38	1.92	48
		0.25	1.05	54	2.38	2.27	39
		0.1	2.31	—	7.48	3.24	13
4	Malonate (30 mm) + NAD (1 mm) + $MnCl_2$ (1 mm)	0.0	2.35	—	8.58	3.65	—
		1.0	0.82	65	0.57	0.69	81

Fig. 1. Polarographic records of the rate of oxygen uptake by rat-heart mitochondria respiring with α -oxoglutarate as substrate. The composition of the medium was that given in the Experimental section for experiments on rat-heart mitochondria. The final volume was 1 ml. The additions were made at the times indicated as follows: A, malonate (8 mm); B, orthophosphate (2 mm); C, α -oxoglutarate (4 mm); D, ADP (0.4 mm); X, 3-hydroxyanthranilic acid (0.5 mm).

incubation (Fig. 1b), an instantaneous and time-independent inhibition of oxygen uptake resulted, which on the addition of ADP did not pass to 'active state 3'. The extent of inhibition was higher after the addition of ADP, thus giving the impression that the respiration was more sensitive to the action of 3-hydroxyanthranilic acid when it occurs in 'active state 3'. 3-Hydroxyanthranilic acid (0.1 mm) gave a negligible inhibition of respiration. At this concentration of 3-hydroxyanthranilic acid it was possible to observe a lowering of the ADP: oxygen ratio, as calculated from the amount of ADP added and the amount of oxygen utilized during the active state of respiration. The respiratory control was not influenced by 0.1 mm-3-hydroxyanthranilic acid, i.e. respiration accelerated by the addition of ADP returned to its initial rate when ADP was expended owing to the oxidative phosphorylation. These results suggested the study of the effect of 3-hydroxyanthranilic acid on respiration and oxidative phosphorylation of mitochondria with succinate as the respiratory substrate. In the experiments in which the oxygen uptake was measured by the manometric method, respiration supported by succinate appeared to remain unaffected by 1 mm-3-hydroxyanthranilic acid. Only a slight inhibition of phosphorylation was observed (Table 2). With the oxygen electrode we measured the rate of oxygen uptake by rat-heart mitochondria oxidizing succinate either in freshly prepared mitochondria or after 15 min. of preincubation. With the latter the oxidation of succinate requires the addition of ATP, in accordance with the results of Azzone & Ernster (1961) and Klingenberg & Schollmeyer (1961) (Fig. 2a).

Table 2. *Effect of 3-hydroxyanthranilic acid on respiration and oxidative phosphorylation of rat-liver mitochondria respiring with succinate as respiratory substrate*

The results refer to 1 ml. of mitochondrial suspension (3–4 mg. of protein). The composition of the medium was that given in the Experimental section for experiments on oxidative phosphorylation. The incubation time was 15 min.

Concn. of 3-hydroxyanthranilic acid (mm)	Oxygen uptake (μ g.atoms)	Phosphate esterified (μ moles)	P:O ratio	Inhibition of phosphorylation (%)
0.0	5.57	11.20	2.01	—
1.0	5.73	9.39	1.64	18
0.1	5.61	11.10	1.97	2

Fig. 2. Polarographic records of rate of oxygen uptake by rat-heart mitochondria respiring with succinate as substrate, after 15 min. of preincubation. The composition of medium was that given in the Experimental section for experiments on rat-heart mitochondria. The additions were made at the times indicated as follows: A, succinate (4 mM); B, ATP (1 mM); C, 2,4-dinitrophenol (0.1 mM); X, 3-hydroxyanthranilic acid (1 mM).

3-Hydroxyanthranilic acid (1 mM) did not significantly affect the rate of succinate oxidation activated by ATP (Fig. 2b).

Effect of 3-hydroxyanthranilic acid on the mitochondrial nicotinamide-adenine dinucleotide-dependent systems. Experiments were performed in which the effect of 3-hydroxyanthranilic acid on the oxidative phosphorylation of mitochondria oxidizing, separately, pyruvate plus fumarate,

malate, β -hydroxybutyrate, glutamate, and isocitrate was studied. 3-Hydroxyanthranilic acid (1 mM) inhibited oxygen uptake in all cases. Further, 3-hydroxyanthranilic acid always exerted a more severe inhibition on the phosphate uptake. When isocitrate, β -hydroxybutyrate or malate was used as substrate, NAD (1 mM) was able to restore the inhibited oxygen uptake. The increased respiratory rate was not followed by a corresponding increase in phosphate uptake. In this case we had a true uncoupling effect. When the mitochondrial respiration was supported by glutamate or pyruvate plus fumarate, NAD could not completely restore the inhibited rate of oxygen uptake. The respiration of mitochondria in the presence of these substrates is partly due to the oxidation of α -oxoglutarate that is formed from glutamate by transamination of the amino acid with oxaloacetate or by oxidative deamination, and from pyruvate plus fumarate by operation of the first steps of the citric acid cycle. The results are reported in Table 3. Figs. 3 and 4 show the effect of 1 mM-3-hydroxyanthranilic acid on the respiration of mitochondria in the presence of β -hydroxybutyrate and α -oxoglutarate during the time of incubation. The inhibitory effect of 3-hydroxyanthranilic acid on the oxidation of β -hydroxybutyrate appeared to be progressive with the time of incubation and always reversible by the addition of NAD. The inhibition of α -oxoglutarate oxidation was instantaneous, linear with time and not reversed by NAD.

Effect of 3-hydroxyanthranilic acid on the oxidation of α -oxoglutarate in the presence of 2,4-dinitrophenol. The oxidation of α -oxoglutarate by uncoupled mitochondria is controlled by the availability of orthophosphate to the same extent as the oxidation by tightly coupled mitochondria is controlled by ADP (Azzone & Ernster, 1961; Borst & Slater, 1961; Papa, Budillon & Quagliariello, 1962a). We observed this phenomenon both in rat-liver mitochondria (manometric measurements, see Figs. 5 and 6) and in rat-heart mitochondria (polarographic records, see Figs. 1a and 7a). 3-Hydroxyanthranilic acid completely suppressed the activation of the

Table 3. Effect of 3-hydroxyanthranilic acid on respiration and oxidative phosphorylation of rat-liver mitochondria in the presence of nicotinamide-adenine dinucleotide-dependent respiratory substrates

The results refer to 1 ml. of mitochondrial suspension (3-4 mg. of protein). The composition of medium was that given in the Experimental section for experiments on oxidative phosphorylation. The incubation time was 15 min.

Expt. no.	Substrate added	NAD (1 mM)	Control (3-hydroxyanthranilic acid absent)				3-Hydroxyanthranilic acid (1 mM) present			
			Oxygen uptake (μg.atoms)	Phosphate esterified (μmoles)	P:O ratio	Inhibition of respiration (%)	Oxygen uptake (μg.atoms)	Phosphate esterified (μmoles)	P:O ratio	Inhibition of phosphorylation (%)
1	L-Malate	-	2.24	5.29	2.36	41	1.32	0.25	0.19	92
		+	3.47	8.62	2.48	—	3.67	3.02	0.82	66
2	DL-Isocitrate	-	5.24	14.95	2.85	41	3.05	3.15	1.03	63
		+	5.67	16.17	2.85	—	5.55	7.88	1.42	50
3	DL-β-Hydroxybutyrate	-	2.56	6.95	2.71	46	1.38	1.54	1.12	58
		+	2.93	8.16	2.78	—	3.39	5.44	1.60	42
4	L-Glutamate	-	5.32	15.3	2.88	53	2.52	1.95	0.77	73
		+	5.84	16.5	2.82	33	3.91	6.95	1.77	37
5	Pyruvate + fumarate	-	5.48	15.85	2.89	56	2.40	2.83	1.18	59
		+	5.54	16.30	2.94	16	4.65	6.28	1.35	54

oxygen uptake either by ADP in coupled mitochondria or by orthophosphate in the presence of dinitrophenol. The results suggest that the inhibition observed in the two conditions is due to a unique effect of 3-hydroxyanthranilic acid on the dehydrogenase complex, probably on the α-oxoglutarate-linked substrate phosphorylation.

Effect of tryptophan metabolites on mitochondrial oxidative phosphorylation. Table 4 reports the results of a series of experiments in which the effect of tryptophan, kynurenine, xanthurenic acid, kynurenic acid, anthranilic acid and 3-hydroxykynurenine on the mitochondrial oxidative phosphorylation in the presence of α-oxoglutarate plus malonate was studied. Of all the compounds tested, besides 3-hydroxyanthranilic acid, only anthranilic acid and kynurenic acid had a slight effect on oxygen uptake.

Effect of 3-hydroxyanthranilic acid on mitochondrial adenosine triphosphatase. The addition of 3-hydroxyanthranilic acid to intact rat-liver mitochondria resulted in an activation of the normal adenosine triphosphatase. However, the dinitrophenol-induced adenosine triphosphatase was not influenced by 3-hydroxyanthranilic acid (Fig. 8). The 3-hydroxyanthranilic acid-induced adenosine-

Fig. 3. Effect of 3-hydroxyanthranilic acid on the oxidation of β-hydroxybutyrate. The oxygen consumptions refer to 1 ml. of mitochondrial suspension (3-4 mg. of protein). The composition of medium was that given in the Experimental section for experiments on oxidative phosphorylation. O, Control (no addition); ●, 3-hydroxyanthranilic acid (1 mM) present; ■, NAD (1 mM) plus 3-hydroxyanthranilic acid (1 mM) present.

triphosphatase activity did not require Mg^{2+} ions and was almost completely inhibited by the presence of succinate in substrate quantities; β -hydroxybutyrate and α -oxoglutarate were less effective (see Table 5).

3-Hydroxyanthranilic acid and mitochondrial structural state. The effect of 3-hydroxyanthranilic acid on mitochondrial structure was examined by measuring ΔE_{520} of mitochondrial suspensions. 3-Hydroxyanthranilic acid (1 mM) added to diluted mitochondrial suspensions (1–0.75 mg. of protein/3 ml.) at 30° did not show, in an interval of 30 min., any lytic or large-amplitude-swelling effect on mitochondria (see Fig. 9). It did, however, increase the spontaneous swelling of mitochondria.

DISCUSSION

The results reported above show that, of all the tryptophan metabolites tested, only 3-hydroxyanthranilic acid produces definite effects on mitochondrial respiration and oxidative phosphorylation. The pattern of 3-hydroxyanthranilic acid action on mitochondrial processes may be summarized as follows: (1) it inhibits the oxygen uptake by mitochondria respiring with NAD-dependent substrates; (2) it inhibits the respiratory-

chain phosphorylation, showing in some cases an uncoupling effect; (3) it activates the normal adenosine triphosphatase of intact mitochondria without affecting the dinitrophenol-induced adenosine-triphosphatase activity.

The most severe inhibition of oxygen uptake by 3-hydroxyanthranilic acid (about 80% at a 3-hydroxyanthranilic acid concentration of 1 mM) occurred when the respiration of rat-liver or rat-heart mitochondria was supported by α -oxoglutarate. When the mitochondrial respiration is supported by α -oxoglutarate as substrate, the oxygen uptake is the result of the oxidation of this compound plus the further oxidation of succinate produced by α -oxoglutarate decarboxylation. To investigate the effect of 3-hydroxyanthranilic acid on oxoglutarate oxidation when limited to the first step, in some experiments (Table 1: Expts. 2 and 3) an excess of malonate was added to specifically inhibit succinate dehydrogenase. The finding that 3-hydroxyanthranilic acid inhibited the oxygen uptake at the same percentage in the presence or absence of malonate, and inhibited the phosphate

Fig. 4. Effect of 3-hydroxyanthranilic acid on the oxidation of α -oxoglutarate. The oxygen consumptions refer to 1 ml. of mitochondrial suspension (3–4 mg. of protein). The composition of medium was that given in the Experimental section for experiments on oxidative phosphorylation. ○, Control (no addition); ●, 3-hydroxyanthranilic acid (1 mM) present.

Fig. 5. Effect of 3-hydroxyanthranilic acid on the activation by ADP of the oxidation α -oxoglutarate in coupled rat-liver mitochondria. The oxygen consumptions refer to 1 ml. of mitochondrial suspension (3–4 mg. of protein). The composition of the medium was that given in the Experimental section for experiments on oxidative phosphorylation. □, ATP-glucose-hexokinase system absent; ○, ATP-glucose-hexokinase system present; ●, ATP-glucose-hexokinase system plus 3-hydroxyanthranilic acid (1 mM) present; ■, ATP-glucose-hexokinase system absent, 3-hydroxyanthranilic acid (1 mM) present.

uptake at a percentage twice that found in the absence of malonate, shows that 3-hydroxyanthranilic acid acts on the first step of α -oxoglutarate oxidative phosphorylation, without directly affecting the succinate oxidative phosphorylation. This argument is directly supported by the lack of inhibition of respiration by 3-hydroxyanthranilic acid when succinate was the substrate. The results of these experiments suggest that the compound acts on the processes of mitochondrial respiration before the point where cytochrome *b* comes into play; this point is commonly regarded as the entrance of electron transport from NADH_2 and succinate dehydrogenase into the cytochrome system. We can distinguish two cases for the inhibition of the oxidation of other NAD-dependent substrates. When malate, isocitrate or β -hydroxybutyrate was used as substrate, the oxygen uptake was inhibited by about 40% by 1 mM-3-hydroxyanthranilic acid. The inhibitory effect of 3-hydroxyanthranilic acid appeared to be time-dependent and was prevented by the presence of NAD in the incubation medium. In contrast the

Fig. 6. Effect of 3-hydroxyanthranilic acid on the activation by orthophosphate of the oxidation of α -oxoglutarate in 2,4-dinitrophenol-uncoupled rat-liver mitochondria. The oxygen consumptions refer to 1 ml. of mitochondrial suspension (3–4 mg. of protein). The composition of medium was that given in the Experimental section for experiments on respiration in the presence of 2,4-dinitrophenol. ○, Orthophosphate absent; ●, orthophosphate present; ▲, orthophosphate plus 3-hydroxyanthranilic acid (1 mM) present; △, orthophosphate absent, 3-hydroxyanthranilic acid (1 mM) present.

Fig. 7. Polarographic records of the rate of oxygen uptake by rat-heart mitochondria respiring with α -oxoglutarate as substrate. The composition of the medium was that given in the Experimental section for experiments on rat-heart mitochondria. The final volume was 1 ml. The additions were made at the times indicated as follows: A, malonate (8 mM); B, 2,4-dinitrophenol (0.1 mM); C, α -oxoglutarate (4 mM); D, orthophosphate (2 mM); X, 3-hydroxyanthranilic acid (1 mM).

Table 4. Effect of tryptophan metabolites on respiration and oxidative phosphorylation of rat-liver mitochondria in the presence of α -oxoglutarate plus malonate

The results refer to 1 ml. of mitochondrial suspension (3–4 mg. of protein). The composition of medium was that given in the Experimental section for experiments on oxidative phosphorylation. The incubation time was 20 min.

Compound added (1 mM)	Oxygen uptake ($\mu\text{g. atoms}$)	Phosphate esterified (μmoles)	P:O ratio
None	3.30	11.55	3.5
DL-Tryptophan	3.40	10.88	3.2
DL-Kynurenine	3.42	10.94	3.2
Xanthurenic acid	3.12	9.67	3.1
Kynurenic acid	2.95	9.73	3.3
Anthranilic acid	2.55	7.83	3.1
3-Hydroxy-DL-kynurenine	3.02	9.36	3.1
3-Hydroxyanthranilic acid	0.69	0.34	0.5

inhibition of α -oxoglutarate oxidation was immediate, linear with time and not prevented by NAD. These findings suggest that: (1) the inhibition of the oxidation of malate, isocitrate and β -hydroxybutyrate is the consequence of a loss of mitochondrial NAD; (2) 3-hydroxyanthranilic acid does not act directly on the respiratory chain; (3) the inhibition of α -oxoglutarate oxidation by 3-hydroxyanthranilic acid is, besides the loss of NAD, also dependent on the action of 3-hydroxyanthranilic acid at the dehydrogenase-complex level, at a point before the respiratory chain. This is consistent with the finding that, when 3-hydroxyanthranilic acid is added to dinitrophenol-uncoupled mitochondria oxidizing α -oxoglutarate, the activating effect of orthophosphate on respiration is suppressed. In agreement with Azzone & Ernster (1961) and Borst & Slater (1961), we found that the activation by orthophosphate of the respiration of rat-liver and rat-heart mitochondria was uncoupled by dinitrophenol, and could demonstrate directly that the control of uncoupled oxidation of α -oxoglutarate by the availability of orthophosphate is of the same order of magnitude as the control of respiration of tightly coupled mitochondria by ADP. We agree with Azzone & Ernster (1961) and Borst & Slater (1961) that this

Fig. 8. Effect of 3-hydroxyanthranilic acid on the normal adenosine triphosphatase of intact mitochondria and on 2,4-dinitrophenol-induced adenosine-triphosphatase activity. The values of orthophosphate liberated refer to 2 ml. of mitochondrial suspension (1.5–2 mg. of protein). The composition of the medium was that given in the Experimental section for experiments on mitochondrial adenosine triphosphatase. ○, Control (no addition); ●, 3-hydroxyanthranilic acid (1 mM) present; △, 2,4-dinitrophenol (0.1 mM) present; ▲, 2,4-dinitrophenol (0.1 mM) plus 3-hydroxyanthranilic acid (1 mM) present.

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Table 5. Effect of magnesium ions and respiratory substrates on 3-hydroxyanthranilic acid-induced adenosine-triphosphatase activity

The results refer to 2 ml. of mitochondrial suspension (1.5–2 mg. of protein). The composition of the medium was that given in the Experimental section for experiments on mitochondrial adenosine triphosphatase. The incubation time was 25 min.

Additions	Orthophosphate liberated (μmoles)	
	Control (3-hydroxyanthranilic acid absent)	3-Hydroxyanthranilic acid (1 mM) present
None	0.90	3.90
Mg ²⁺	1.70	3.95
Mg ²⁺ + succinate	1.01	0.92
Mg ²⁺ + β -hydroxybutyrate	0.88	2.12
Mg ²⁺ + α -oxoglutarate	0.83	1.98

Fig. 9. Effect of 3-hydroxyanthranilic acid on the stability of rat-mitochondria suspensions. Each cuvette contained, in a final volume of 3 ml., mitochondria from 50 mg. of liver. The compositions of the media were: ○, KCl (0.125M) in 20 mM-tris buffer, pH 7.4; ●, KCl (0.125M) and 3-hydroxyanthranilic acid (1 mM) in 20 mM-tris buffer, pH 7.4; △, KCl (0.0625M) in 20 mM-tris buffer, pH 7.4. The temperature was 25°.

feature of α -oxoglutarate oxidation, which is not shared by other respiratory substrates, is due to α -oxoglutarate-linked phosphorylation at the substrate level. When the mitochondrial respiration was supported by glutamate or by pyruvate plus fumarate, the inhibition of oxygen uptake was more marked (about 60 % at a 3-hydroxyanthranilic acid concentration of 1 mM), and NAD was not able to completely prevent the inhibitory effect. Thus the inhibition of respiration supported by these substrates appears to be partly due (for the percentage of inhibition which is removed by NAD) to the loss of NAD, and partly to the inhibition of the oxidation of the α -oxoglutarate that is formed and oxidized in these systems. As far as the effect of 3-hydroxyanthranilic acid on oxidative phosphorylation is concerned, it was found that, when NAD-dependent respiratory substrates were used, the orthophosphate uptake was always more severely inhibited than the oxygen consumption.

It must be stressed that: (1) the increased respiratory rate observed on the addition of NAD to the 3-hydroxyanthranilic acid-treated mitochondrial suspension was not followed by a corresponding increase in the orthophosphate uptake. In this case we could demonstrate a true uncoupling effect, the P:O ratios being almost 1 unit lower than the values of control experiments; (2) the addition of an excess of malonate to mitochondria oxidizing α -oxoglutarate led to the percentage inhibition of oxidative phosphorylation, now supported only by α -oxoglutarate oxidation, being approximately twice as high as in the absence of malonate; (3) with succinate as respiratory substrate, 3-hydroxyanthranilic acid exerted only a slight inhibition of orthophosphate uptake. From these results it appears conceivable that the first respiratory-chain phosphorylation is more sensitive to the action of 3-hydroxyanthranilic acid.

3-Hydroxyanthranilic acid activates immediately and linearly with time the adenosine triphosphatase of intact mitochondria. The adenosine-triphosphatase activity so induced appears to be virtually independent of added Mg^{2+} ions. This result, together with the lack of any effect of 3-hydroxyanthranilic acid on dinitrophenol-induced adenosine-triphosphatase activity, indicates a similarity between the site of action of the two substances.

It has been postulated by Löw, Siekevitz, Ernster & Lindberg (1958) that the dinitrophenol-induced adenosine-triphosphatase activity is related to the first of the three oxidative-phosphorylation sequences, namely that which occurs in the NAD-flavin region. It would therefore seem that the effect of 3-hydroxyanthranilic acid concerns the first phosphorylation step. The results on the effect of the addition of respiratory substrates on

the adenosine-triphosphatase activity should also be considered. Though succinate was able to inhibit completely the 3-hydroxyanthranilic acid-induced adenosine triphosphatase, β -hydroxybutyrate and α -oxoglutarate were only partially effective. In the absence of added 3-hydroxyanthranilic acid all three substrates had the same inhibitory effect. These results are consistent with the conclusion that 3-hydroxyanthranilic acid interferes with the first phosphorylation step, leaving unaffected the other two respiratory-chain phosphorylation steps in the cytochrome region. Chance & Ito (1963) suggested that the inhibition of adenosine triphosphatase in tightly coupled mitochondria is due to the binding by $NADH_2$ of one of the intermediates of the energy-transfer sequence (the most proximal to the respiratory chain being in the NAD-flavin region). A target of 3-hydroxyanthranilic acid at this level could explain, at the same time, the activation of adenosine triphosphatase, the loss of mitochondrial NAD, with the inhibition of the dependent oxidations, and also the uncoupling effect of 3-hydroxyanthranilic acid.

SUMMARY

1. 3-Hydroxyanthranilic acid (1–0.25 mM) inhibits the oxygen uptake of rat-liver and rat-heart mitochondria respiring with NAD-dependent substrates. This appears to be due to loss of mitochondrial NAD, and to inhibition of the α -oxoglutarate-dehydrogenase complex. 3-Hydroxyanthranilic acid does not appear to act directly on the respiratory chain.

2. 3-Hydroxyanthranilic acid completely suppresses the activation of α -oxoglutarate oxidation either by ADP in tightly coupled mitochondria or by orthophosphate in the presence of 2,4-dinitrophenol.

3. When NAD-dependent respiratory substrates were used, the orthophosphate uptake was always more severely inhibited by 3-hydroxyanthranilic acid than was the oxygen consumption. The addition of NAD prevented the inhibition of NAD-dependent oxidations (except for α -oxoglutarate oxidation), but there was no corresponding increase in orthophosphate uptake.

4. 3-Hydroxyanthranilic acid activates the normal adenosine triphosphatase of intact mitochondria, but not the dinitrophenol-induced adenosine-triphosphatase activity.

5. These effects of 3-hydroxyanthranilic acid seem to be explained by action of the substance at the level of the first respiratory chain phosphorylation.

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Composition of Tissue Lipids and Anaemia of Guinea Pigs in Response to Dietary Cholesterol

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The guinea pig is unusual among experimental animals in that it reacts to feeding with cholesterol by an accelerated destruction of erythrocytes, splenic enlargement and hyperplasia of bone marrow (Okey & Greaves, 1939; Kennedy & Okey, 1947; Okey, 1944). As a result, death usually occurs before any considerable atherosclerotic plaques are formed.

The possibility that the characteristic response of the guinea pig to cholesterol may result from changes in the lipid composition of the erythrocytes or their environment, or both, deserved investigation. The present paper reports fatty acid compositions of the lipid fractions from plasma, erythrocytes, livers and spleens of control and cholesterol-fed guinea pigs.

MATERIALS AND METHODS

Male and female guinea pigs, weighing 225–290 g. at 2–3 weeks of age, were used (Dependable Animal Supply Co., Martinez, Calif.). They were housed individually and

fed *ad libitum* on a semi-synthetic diet similar to that used by Richie (1959). For the first 2 weeks all were given the control diet, composed of the following (g./100 g. of diet): casein, 20; albumin, 10; cottonseed oil, 10; sucrose, 7; glucose, 6.8 (Cerelease from Corn Products, San Francisco, Calif.); dextrin, 20; cellophane spangles, 5 (from Rayon Processing Co., Pawtucket, R.I.); alfalfa meal, 5; agar, 5; salt mix, 4 (USP XIV; zinc carbonate was incorporated to give a concentration of 0.17% in the diet); magnesium oxide, 0.4; potassium acetate, 2; choline bitartrate, 0.36 (choline, 0.17); ascorbic acid, 0.2; inositol, 0.2; vitamin A mix, 2.0 [vitamin A mix contained (g./kg. of mix): vitamin A acetate, 0.3; viosterol, 0.2 (Nutritional Biochemical Corp.; 400 000 USP units of vitamin D/g.); α -tocopherol, 1; cottonseed oil, 998]; vitamin B mix, 2.0 [vitamin B mix contained (g./kg. of mix): thiamine hydrochloride, 0.8; ascorbic acid, 25; riboflavin, 0.8; pyridoxine hydrochloride, 0.8; calcium pantothenate, 2; nicotinic acid, 10; biotin, 0.1; folic acid, 0.5; glucose, 960]. The fatty acid composition of the diet is shown in Table 1. Analyses of the alfalfa meal and the agar showed that they contained 8.3% and 4.4% of ash respectively. These ashes were shown to contain less than 1% of magnesium (both) and 8.8% and 4.6% of