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IN VITRO EFFECTS OF NONSTEROIDAL ANTI-INFLAMMATORY DRUGS ON OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA

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Abstract—Representative potent anti-inflammatory drugs have been studied for their ability to affect energy metabolism *in vitro* in rat liver mitochondria. Like 2,4-dinitrophenol, flufenamic acid activated ATPase, inhibited the incorporation of ^{32}Pi into the ATP fraction, stimulated glutamate oxidation and coupled substrate-level phosphorylation, and promoted state-4 respiration. Aspirin and ibuprofen caused similar but lesser effects on these parameters. These three drugs are considered to be uncouplers of oxidative phosphorylation. Indomethacin and phenylbutazone inhibited both oxidative and substrate-level phosphorylations. Based on their abilities to inhibit glutamate oxidation rather directly and to block the electron transport chain, it was concluded that indomethacin and phenylbutazone could inhibit the generation of energy-rich phosphate compounds by suppressing respiration at multiple sites. Thus, these anti-inflammatory drugs could prevent mitochondrial energy metabolism in different manners.

Although anti-inflammatory drugs form a very heterogeneous group, it is well known that these drugs have many biochemical properties in common, one of which is the uncoupling of oxidative phosphorylation. The uncoupling action of anti-inflammatory drugs was studied first by Adams and Cobb [1], and later in more detail by several investigators [2–6]. The present paper describes *in vitro* effects of several anti-inflammatory drugs having different structures on oxidative phosphorylation and related energy metabolism in isolated rat liver mitochondria. It is shown that these drugs affected oxidative phosphorylation in somewhat different manners.

MATERIALS AND METHODS

Rat liver mitochondria were isolated by the method of Johnson and Lardy [7] in 0.33 M sucrose–0.1 mM EDTA solution. The standard reaction mixture contained 146 mM KCl, 20 mM Tris–HCl buffer (pH 7.4), 1 mM EDTA, 55 mM sucrose (derived from mitochondrial suspension) and mitochondria from 25 mg rat liver (containing 0.6–0.65 mg protein) in a final volume of 1.0 ml. This reaction mixture was incubated at 25°. When oxygen utilization was estimated with Clark oxygen electrode, the reaction mixture was scaled to 10-times this quantity with mitochondria from 500 mg rat liver.

Determination of ATPase activity. Mitochondria were incubated in the standard reaction mixture added with 1 mM ATP. The incubation was terminated by the addition of 0.2 ml of 7% $HClO_4$ and the deproteinized supernatant was assayed for inorganic phosphate (Pi) by the method of Fiske–Subbarow [8]. Without further additions essentially no Pi was liberated during incubation. When 2,4-dinitrophenol(DNP) was added at concentrations up to 0.1 mM, Pi accumulation occurred linearly for the first 15 min as a result of breakdown of ATP. Therefore, a 15-min incubation was carried out to estimate the activity of ATPases stimulated by DNP and anti-inflammatory drugs.

Incorporation of ^{32}Pi into mitochondrial adenine nucleotides. Mitochondria were incubated for 2.5 min in the standard reaction mixture added with 0.05 mM ^{32}Pi. Separation of adenine nucleotides labelled with ^{32}P during incubation was carried out on the thin-layer plate of polyethyleneimine–cellulose [9] after treatment of the acidified (deproteinized) medium with charcoal according to the procedure described elsewhere in detail [10]. When mitochondria were incubated with tetramethyl-*p*-phenylenediamine(TMPD), ascorbate and rotenone, ^{32}Pi was incorporated into ATP almost exclusively. In this case, therefore, ^{32}Pi was precipitated off by the method of Sugino and Miyoshi [11] and the supernatant was counted for ^{32}P as a simple and tentative measure of $AT^{32}P$ [12].

$^{14}CO_2$ liberation from [1-^{14}C]glutamate. Our previous studies showed that the production of $^{14}CO_2$ from [1-^{14}C]glutamate reflects the oxidation rate of glutamate in the mitochondria rather than the penetration rate of glutamate into mitochondria if the concentration of glutamate in the medium was maintained above 1 mM [10]. Accordingly, a mitochondrial suspension added with 1 mM [1-^{14}C]glutamate was incubated for 20 min in the rubber-stoppered vial equipped with a suspending plastic cup. The reaction was terminated by injecting 0.25 ml of $HClO_4$ through the rubber stopper along the wall of the vial to make a final concentration of 0.5 M. Following further addition of hyamine onto a roll of paper in the centre cup, the vial was stored at room temperature for 1 to 2 hr. Radioactivity trapped by hyamine was then measured.

Table 1. Kinetic parameters of ATPases stimulated by DNP and anti-inflammatory drugs

Drugs	K_a(M)	V_{max} (nmole Pi/mg protein/min)
DNP	1×10^{-5}	87
Flufenamic acid	2×10^{-6}	46
Indomethacin	8×10^{-5}	49
Ibuprofen	4×10^{-5}	18
Phenylbutazone	6×10^{-4}	33

The data in this and the following tables are the means from two observations.

Oxygen consumption. Oxygen consumption was measured polarographically with a Clark oxygen electrode [13]. Some experiments were carried out with the damaged mitochondria prepared by exposing suspensions of intact mitochondria to one or two cycles of freezing and thawing.

Chemicals. Representative anti-inflammatory drugs were used: flufenamic acid as one of anthranilic acid derivatives, indomethacin as an indoleacetic acid derivative, ibuprofen as a phenylacetic acid derivative, phenylbutazone and aminopyrine as pyrazoline derivatives and acetyl salicylate (aspirin) as a salicylate. These drugs are generous gifts from Dr. K. Matsuki, Lederle (Japan) Ltd. Other reagents were of the purest grade commercially available. Stock solutions of aspirin and aminopyrine were prepared by dissolving the drugs in Tris–HCl buffer (pH 7.4). Other anti-inflammatory drugs were dissolved in ethanol and added to the reaction mixture after dilution with buffer. As a result, the concentration of ethanol in the reaction mixture was 0.1 per cent for all drugs other than phenylbutazone which was added to the reaction mixture with ethanol at the final concentration of 0.5 per cent. The mitochondrial suspension added with 0.1 or 0.5 per cent ethanol alone was used as control, though ethanol in amounts smaller than 0.5 per cent did not cause detectable effects on mitochondrial reactions measured in the present study.

RESULTS

Activation of ATPase by anti-inflammatory drugs. Anti-imflammatory drugs activated ATPase when added to mitochondrial suspensions. V_{max} of the ATPases thus activated, and the concentrations of these drugs required for inducing the half-maximal activity (K_a), were estimated based on double reciprocal plots of the initial velocities (not shown), and are summarized in Table 1 together with the kinetic parameters of DNP-stimulated ATPase obtained under the same conditions. DNP-stimulated ATPase was much more active than the enzymes stimulated by anti-inflammatory drugs. Among the anti-inflammatory drugs tested, flufenamic acid and indomethacin were the strongest ATPase activators, though a much higher concentration of the latter was required for the same activation than the former. Phenylbutazone, when added at one- or two-orders of magnitude higher concentrations than others, caused less active ATPase. Ibuprofen stimulated ATPase only slightly.

ATPases activated by these anti-inflammatory drugs, like DNP-activated ATPase, were inhibited by 1 μM oligomycin (Table 2), indicating that ATP decomposed in the presence of these drugs via the oligomycin-sensitive oxidative phosphorylation pathway. Oligomycin inhibited ATPase in an almost non-competitive manner. The K_i for the oligomycin inhibition was calculated according to Dixon and Webb [14] and also presented in Table 2. The affinity of enzyme to oligomycin was essentially the same among ATPases activated by DNP, flufenamic acid and ibuprofen. In contrast, indomethacin-stimulated ATPase was inhibited more efficiently and phenylbutazone-stimulated ATPase was inhibited less efficiently by oligomycin. The results might suggest that these two drugs activate ATPase in a manner somewhat different from DNP-activated ATPase.

Incorporation of ^{32}Pi into adenine nucleotides. As a measure of phosphorylation, the incorporation of ^{32}Pi into ATP and ADP was determined in the pres-

Table 2. Inhibition by oligomycin of ATPases stimulated by DNP and anti-inflammatory drugs

Drugs	ATPase activity*		
	Without	With oligomycin (1 μM)	K_i†
	(nmole Pi/mg protein/min)		
DNP (10 μM)	52	20	6×10^{-7}
Flufenamic acid (10 μM)	45	14	5×10^{-7}
Indomethacin (0.1 mM)	34	4	1×10^{-7}
Ibuprofen (0.1 mM)	15	6	5×10^{-7}
Phenylbutazone (0.5 mM)	26	15	1×10^{-6}

* ATPase activity: "Pi released with drug" minus "Pi released without drug".

† K_i = Concentration of oligomycin $\times$ $(v_i/(v - v_i))$, where v is ATPase activity without oligomycin and v_i is ATPase activity with oligomycin.

Table 3. Effects of DNP and anti-inflammatory drugs on the incorporation of ^{32}Pi into adenine nucleotides under various conditions

Drug	System 1	System 2		System 3		System 4
	ATP	ATP	ADP	ATP	ADP	ATP
	(cpm × 10^{-3}/mg protein)					
None	232	64	98	396	429	72
DNP (10 μM)	129	30	77	251	653	6
(50 μM)	3	11	64	227	597	6
Flufenamic acid (10 μM)	58	32	66	372	537	7
Indomethacin (0.1 mM)	6	5	29	100	285	6
Ibuprofen (0.1 mM)	126	54	82	292	384	33
Phenylbutazone (0.5 mM)	26	8	37	99	146	10
Aspirin (1 mM)	230	45	134	341	629	5
Aminopyrine (1 mM)	218	44	98	422	416	77

System 1: added with 10 mM succinate and 1 mM ATP.
System 2: added with 10 mM glutamate.
System 3: added with 10 mM glutamate and 0.1% albumin.
System 4: added with 0.14 mM N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD), 3 mM ascorbate and 0.2 μM rotenone.

ence of DNP or anti-inflammatory drugs (Table 3). With succinate and ATP (system 1 in Table 3), ^{32}Pi was incorporated into the ATP fraction almost exclusively (92% $AT^{32}P$ and 8% $AD^{32}P$ in the total ^{32}P-labeled nucleotides). The ^{32}Pi-ATP exchange reaction in the presence of succinate was inhibited by indomethacin and phenylbutazone as strongly as by 50 μM DNP. It was also inhibited by flufenamic acid significantly but less markedly. Ibuprofen was a weak inhibitor, and aspirin and aminopyrine were without effect, in this system.

When a mitochondrial suspension was incubated with glutamate without addition of Pi acceptor, ^{32}Pi was incorporated into ADP as well as ATP. Our previous reports [10, 12, 15, 16] provided evidence that, under these conditions, $AT^{32}P$ is formed via oxidative phosphorylation while $AD^{32}P$ is formed via the substrate-level phosphorylation coupled to oxidation of glutamate. Consequently, the addition of uncouplers of oxidative phosphorylation inhibited $AT^{32}P$ formation but did not inhibit $AD^{32}P$ formation [10]. The data under the heading of 'system 2' in Table 3 show that $AT^{32}P$ formation in the presence of glutamate was inhibited not only by DNP but also by most of anti-inflammatory drugs; their relative potency as inhibitors in system 2 was essentially the same as observed for the inhibition of ^{32}Pi-ATP exchange reaction in the presence of succinate (system 1). In contrast, the phosphorylation of AMP ($AD^{32}P$ formation) was not significantly inhibited by DNP and uncouplers other than indomethacin and phenylbutazone.

In system 3 in Table 3, bovine serum albumin was further added to the glutamate-containing medium. It was previously shown [10, 12] that, in this system, the uncoupling action of DNP was too weak to reduce $AT^{32}P$ content markedly but was still effective in accelerating electron flow along the respiratory chain, resulting in increased $AD^{32}P$ formation due to oxidation of glutamate. It is shown in Table 3 that not only DNP but also flufenamic acid and aspirin increased $AD^{32}P$ content after incubation of mitochondria in system 3. The addition of indomethacin or phenylbutazone, however, were still very inhibitory to $AD^{32}P$ formation, strongly suggesting that these anti-inflammatory drugs act on mitochondrial phosphorylation reactions by a mechanism distinct from uncouplers such as DNP.

When a mitochondrial suspension was incubated with TMPD plus ascorbate as an electron donor in the presence of rotenone which blocks the oxidation of NAD-dependent endogenous substrate, $AT^{32}P$ was generated at the coupling site 3 without appreciable formation of $AD^{32}P$. $AT^{32}P$ formation under these conditions was almost totally inhibited by most of drugs tested; it was reduced significantly even by ibu-

Table 4. Effects of DNP and anti-inflammatory drugs on $^{14}CO_2$ production from [1-^{14}C]glutamate

Drugs	Lowest dose	$^{14}CO_2$ production (% of control)		
		Lowest dose	× 10	× 100
DNP	1 μM	144	281	181
Flufenamic acid	0.1 μM	117	166	220
Indomethacin	1 μM	107	127	63
Ibuprofen	10 μM	105	155	167
Phenylbutazone	5 μM	92	112	53
Aspirin	10 μM	108	134	240
Aminopyrine	10 μM	85	100	94

The concentration of [1-^{14}C]glutamate: 1 mM.

Table 5. Effects of DNP and anti-inflammatory drugs on $^{14}CO_2$ production in the presence of electron acceptors

Drug	$^{14}CO_2$ production (cpm × 10^{-2}/mg protein)	
	Without	With $K_3Fe(CN)_6$
None (control)	102	202
DNP (10 μM)	187(183)	226(112)
Flufenamic acid (10 μM)	176(172)	270(134)
Ibuprofen (0.1 mM)	146(143)	246(122)
Aspirin (1 mM)	267(261)	295(146)

Per cent of the respective control is shown in parentheses. The concentration of $K_3Fe(CN)_6$: 10 mM.

profen which was a very weak inhibitor of phosphorylation in other systems. Exceptionally, aminopyrine was without effect on phosphorylation even in system 4.

Stimulation and inhibition of glutamate oxidation by anti-inflammatory drugs. Since the phosphorylation of endogenous AMP coupled to glutamate oxidation was significantly affected by most of the anti-inflammatory drugs tested, the effect of these drugs on glutamate oxidation was next studied more directly by monitoring $^{14}CO_2$ liberation from [1-^{14}C]glutamate. Previous studies from our laboratory [10] have shown that $^{14}CO_2$ production from 1 mM [1-^{14}C]-glutamate is limited by the rate of supply (regeneration) of NAD within the mitochondrion. As a result, the uncoupling of oxidative phosphorylation by DNP led to an increased $^{14}CO_2$ formation as shown in the top of Table 4; 10 μM DNP caused a 3-fold increase in $^{14}CO_2$ production as compared with the control incubation without DNP, but a further increase in DNP concentration up to 100 μM caused a less marked increase probably because an extreme lack of energy-rich phosphates would have prevented ^{14}C-labelled glutamate from entering the mitochondrion.

As is shown in Table 4, flufenamic acid (10 μM) and aspirin (1 mM) were as effective as DNP in increasing glutamate oxidation. Ibuprofen also stimulated glutamate oxidation, though the degree of stimulation was less striking than DNP. In contrast, indomethacin and phenylbutazone inhibited $^{14}CO_2$ liberation at their concentrations that were inhibitory to phosphorylation reaction in Table 3. Aminopyrine was the only one drug that was without effect on glutamate oxidation.

In support of the view that NAD regeneration is limiting the whole process of glutamate oxidation, the addition of an electron acceptor such as ferricyanate gave rise to a marked increase in $^{14}CO_2$ liberation from added [1-^{14}C]glutamate as recorded on the top of Table 5. In the presence of ferricyanate, there were no large increases in $^{14}CO_2$ production with DNP or anti-inflammatory drugs which were effective in increasing $^{14}CO_2$ production in the absence of the electron acceptor. These results in Table 5 strongly suggest that these anti-inflammatory drugs, like DNP, stimulate glutamate oxidation in the same manner as electron acceptors; i.e., by facilitating electron transfer through the respiratory chain.

Table 6 shows that the addition of indomethacin or phenylbutazone to a mitochondrial suspension was very effective in inhibiting glutamate oxidation regardless of whether or not the regeneration of NAD was simultaneously accelerated by electron acceptors or Pi acceptor. Since phenazine methosulfate (PMS) accepts electrons directly from NADH, the results in Table 6 indicate that both drugs can inhibit glutamate oxidation by suppressing either glutamate dehydrogenase directly or the activity of enzymes involved in the metabolism of respiratory substrates such as the citric acid cycle.

Effect of anti-inflammatory drugs on oxygen uptake by mitochondria. The utilization of oxygen by mitochondria maintained in state 4 was accelerated by

Table 6. Effects of indomethacin and phenylbutazone on $^{14}CO_2$ production from [1-^{14}C]glutamate in the presence of various electron acceptors

Electron acceptors	$^{14}CO_2$ production (cpm × 10^{-2}/mg protein)		
	No addition (control)	Indomethacin (0.1 mM)	Phenylbutazone (0.5 mM)
Expt. 1			
None	99	79(80)	53(54)
PMS (1 mM)	273	98(36)	13(5)
Methylene blue (2 μM)	165	91(55)	38(23)
$K_3Fe(CN)_6$ (10 mM)	200	87(43)	12(6)
Expt. 2, with ADP plus Pi (1 mM each)			
None	127	86(68)	68(53)
PMS	320	130(41)	18(5)
Methylene blue	188	103(55)	43(23)
$K_3Fe(CN)_6$	223	106(47)	45(20)

PMS: phenazine methosulfate.

Table 7. Effect of anti-inflammatory drugs on oxygen uptake by intact and damaged mitochondria

Drugs	With glutamate (state 4)	With succinate plus DNP	Damaged mitochondria*
	(% of control)		
DNP (10 μM)	381	—	—
Flufenamic acid (10 μM)	360	106	105
Indomethacin (0.1 mM)	132	33	36
Ibuprofen (0.1 mM)	280	111	—
Phenylbutazone (0.5 mM)	123	46	20
Aspirin (1 mM)	273	105	—
Aminopyrine (1 mM)	100	104	—
Antimycin A (1 μM)	—	0	22

The concentration of glutamate and succinate: 10 mM.
* Damaged mitochondria prepared by freezing and thawing were incubated with 10 mM glutamate and 0.05 mM menadione.

an uncoupler such as DNP (Table 7). Likewise, flufenamic acid, aspirin and ibuprofen were effective in stimulating the state-4 respiration. Indomethacin, phenylbutazone and aminopyrine were without effect, showing that they do not uncouple oxidative phosphorylation. When the transfer of electrons from succinate to oxygen was uncoupled from phosphorylation by DNP, the rapid utilization of oxygen was inhibited by antimycin A totally, and by indomethacin and phenylbutazone very markedly. But, there was no change in oxygen utilization in the presence of flufenamic acid, ibuprofen, aspirin or aminopyrine.

Mitochondria damaged by freezing and thawing exhibited a high rate of oxygen utilization when fortified with glutamate and menadione. This oxygen consumption by damaged mitochondria was also inhibited by indomethacin and phenylbutazone as well as by antimycin A. These results in Table 7 indicate that indomethacin and phenylbutazone directly inhibit the electron transfer along the respiratory chain.

DISCUSSION

In the present study, six representative nonsteroidal anti-inflammatory drugs now widely employed for therapeutic purposes have been tested for their actions *in vitro* on energy-linked metabolisms in rat liver mitochondria. With the exception of aminopyrine which was without effect on any parameter of metabolism tested, they exerted significant influences on oxidation pathways and coupled phosphorylation, though the mechanism underlying their actions was not common but rather diverse, as will be discussed below.

Flufenamic acid, aspirin and ibuprofen behaved just like DNP in inhibiting the incorporation of ^{32}Pi into the ATP fraction dependent on the coupling site 3 (Table 3), in accelerating substrate-level phosphorylation coupled to oxidation of glutamate (Table 3), in promoting glutamate oxidation due to enhanced NAD regeneration (Tables 4 and 5) and in stimulating the state-4 respiration (Table 7). Moreover, flufenamic acid and ibuprofen stimulated ATPase (Table 1) which was inhibited by oligomycin with the same K_i as DNP-activated ATPase (Table 2). The concentration of flufenamic acid that induced half the maximal activity of ATPase (about half the ATPase activity activated by a saturating concentration of DNP) was one-order of magnitude lower than the concentration of DNP required for the half-maximal activation of ATPase. Ten μM of this drug caused changes in all the above-mentioned parameters of energy metabolism in a comparable degree with DNP-induced changes. Thus, flufenamic acid is considered as a very strong uncoupler of oxidative phosphorylation in accord with previous studies [17, 18].

On the other hand, DNP-like actions of ibuprofen and aspirin were detected more strikingly in the stimulation of glutamate oxidation than in the inhibition of phosphorylation of ADP. In fact, they did not cause an appreciable reduction of ^{32}Pi-ATP exchange reaction dependent on added succinate or glutamate (Table 3). In the incubation medium fortified with albumin which is known to attenuate the uncoupling action of DNP [10], DNP inhibited oxidative phosphorylation only slightly (Table 3) but it accelerated glutamate oxidation as markedly as in the medium not added with albumin [10]. It is likely that weak uncouplers such as ibuprofen and aspirin caused a stimulation of glutamate oxidation in a greater degree than an inhibition of phosphorylation. Thus, it is concluded that phosphorylation is uncoupled from oxidation by flufenamic acid strikingly, and by ibuprofen and aspirin slightly, in the same manner as the uncoupling induced by DNP. The relative potency of these uncouplers calculated on the basis of their concentrations required to cause the comparable degree of changes in various parameters tested is as follows in a rough approximation: aspirin 1, ibuprofen 10, DNP 40–50, flufenamic acid 200. Mehlman [19] and Miyahara and Karler [20] reported that aspirin acts *in vitro* as an uncoupler of oxidative phosphorylation in mitochondrial fragments and intact mitochondria from rat liver.

The addition of indomethacin or phenylbutazone into a mitochondrial suspension caused marked inhibitions of both respiration-linked and substrate-level phosphorylations (Table 3). Since uncoupling of respiration from phosphorylation inevitably leads to acceleration of the substrate-level phosphorylation due to rapid oxidation of NADH [10, 12], the simultaneous inhibition of oxidative and substrate-level phosphorylations could not be accounted for solely by an uncoupling effect. In accordance with the view that indomethacin and phenylbutazone exert influences on energy metabolism by other means than

uncoupling, the affinity of oligomycin to the ATPases activated by these two drugs was somewhat different from its affinity to DNP-activated ATPase (Table 2).

The oxidation of glutamate was markedly inhibited by indomethacin or phenylbutazone even when the endogenous NADH, generated in association with glutamate oxidation, was reoxidized more directly by PMS rather than by sequential transfer of electrons along the respiratory chanin to oxygen. It is unlikely that the observed inhibition of glutamate oxidation resulted from suppressed entry of glutamate through the mitochondrial membrane, since there was still an inhibition of glutamate oxidation even in the mitochondrial preparation rendered permeable to added substrates by freezing and thawing (Table 7). Thus, it appears that enzymes directly or indirectly involved in the oxidation of glutamate by NAD is one of the sites of inhibition caused by these two anti-inflammatory drugs.

Furthermore, the antimycin-sensitive transfer of electrons from succinate to oxygen, not linked to phosphorylation, was inhibited by either indomethacin or phenylbutazone (Table 7). Both drugs inhibited phosphorylation at the coupling site 3 (Table 3). These results indicate that the respiratory chain is also inhibited by indomethacin and phenylbutazone. It is concluded, therefore, that indomethacin and phenylbutazone inhibited phosphorylation by suppressing respiration at multiple sites.

"Drug-induced inhibition at higher concentrations" was observed with indomethacin-stimulated ATPase; i.e., ATPase stimulated by 0.5 mM indomethacin was only one-fourth the maximal activity stimulated by 0.1 mM (data not shown). Moreover, DNP failed to stimulate ATPase in the presence of this high concentration of indomethacin. Phenylbutazone and other anti-inflammatory drugs were without effect in this regard. Thus, it appears that indomethacin, in addition to the activity as an inhibitor of mitochondrial respiration possesses an oligomycin-like activity at its higher concentration.

In summary, potent anti-inflammatory drugs tested in the present study could suppress the generation of energy-rich phosphate bonds as a result of uncoupling of oxidative phosphorylation or by other mechanisms. Whether the modification of mitochondrial energy metabolism plays an indispensable role in their ability to suppress inflammation still remains a subject for further investigation.

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