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INHIBITION OF ANAEROBIC GLYCOLYSIS IN BOVINE RETINA EXTRACTS BY SALICYLATE AND ACETYLSALICYLATE

M. T. RINAUDO[1], M. CURTO[1], R. BRUNO[2] and C. PONZETTO[3]
[1]Istituto di Chimica biologica, Facolta' di Medicina e Chirurgia and
[2]Cattedra di Biochimica, Facolta' di Medicina veterinaria, dell'Universita' di Torino, Italy
[3]Department of Biochemistry, College of Physicians and Surgeons of Columbia University, New York, U.S.A.

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Abstract—1. Na salicylate 31 mM inhibits anaerobic glycolysis from glucose in bovine retina extracts. The formation rate of DAP and GAP increases while that of FDP, G6P, F6P and lactate decreases. All the above modifications are almost completely removed by 1.4 mM NAD^+.

2. Bovine retina extracts, preincubated for 1 hr at 0°C with 31 mM Na salicylate show a strongly reduced glycolytic activity. In this system G6P and F6P do accumulate, FDP, DAP, GAP and lactate decrease. These effects are not altered adding 3.5 mM NAD^+ to the preincubation mixture.

3. Acetylsalicylate 31 mM inhibits anaerobic glycolysis in crude retina extracts. As the rate of lactate formation decreases, G6P and F6P do accumulate, while FDP, DAP and GAP diminish.

4. Identical modifications are observed adding the inhibitor directly to the incubation mixture, or preincubating it with the extracts at 0°C for 4 hr. 3.5 mM NAD^+ does not remove the effects of acetylsalicylate.

INTRODUCTION

Salicylate inhibits anaerobic lactate formation from glucose in preparations of rat brain, liver and kidney (Bargoni & Balocco, 1961), and of pigeon gizzard smooth muscle (Sisini *et al.*, 1964). The reactions catalyzed by glucose phosphate isomerase, phosphoglucomutase, fructose biphosphate aldolase, glyceraldehyde phosphate dehydrogenase, pyruvate kinase, lactate dehydrogenase (Bargoni, 1964) and phosphoglycerate kinase (Larsson-Raznikiewicz & Wiksell, 1978) are also inhibited. The phosphotransferase (Larsson-Raznikiewicz & Wiksell, 1978) and dehydrogenase (Einarsson *et al.*, 1974) inhibition seems to involve the formation of an enzyme-salicylate complex by interaction of a carboxyl oxygen of the enzyme with the hydroxyl group of the salicylate. However glyceraldehyde phosphate dehydrogenase is inhibited not only by salicylate but also by acetylsalicylate (Grisolia *et al.*, 1968); in this molecule an acetylic group substitutes for the phenolic hydrogen. Therefore both compounds could act with a mechanism different from the one previously described for salicylate.

In this paper we compare the effects of salicylate and acetylsalicylate on the formation rate from glucose, of lactate and several glycolytic intermediates (glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-biphosphate, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate).

Retina was chosen as an ideal experimental tissue, due to its high glycolytic activity, which only requires the addition of ATP and Mg^{2+}, as opposed to the other tissues that depend upon the addition of exogenous NAD^+ (Rinaudo *et al.*, unpublished results).

MATERIALS AND METHODS

Retinas were obtained from eyes of oxen just killed, kept in ice and used within 1–2 hr.

Salicylate and acetylsalicylic acid were purchased from Merck; ATP and NAD^+ from Sigma.

The extracts were prepared by homogenizing one part of bovine retina in eight parts of 0.1 M Tris–HCl buffer at pH 8, utilizing a Potter homogenizer with Teflon pestle. The homogenate was centrifuged for 60 min in a refrigerated Spinco at 30,000 *g*.

Glycolytic activity was determined in 1 ml samples containing: 0.5 ml supernatant, 13 mM Tris–HCl + 17 mM K_2HPO_4–KH_2PO_4 buffer at pH 7.4, 3.4 mM ATP, 8 mM $MgCl_2$ and 26 mM glucose. The incubation was carried out in Thunberg tubes under vacuum at 37°C. The reaction was stopped after 60 min by adding 1 ml of 6% $HClO_4$. The samples were centrifuged in the cold; 10 N KOH was then added to bring the supernatant to pH 6–6.5. In these samples glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-biphosphate (FDP), dihydroxyacetone phosphate (DAP), glyceraldehyde-3-phosphate (GAP) and lactate were measured spectrophotometrically, as previously described (Rinaudo *et al.*, 1976).

Na salicylate and acetylsalicylate were dissolved and neutralized (pH 8) with 0.1 M Tris, and used at final concentration of 31 mM. In a first set of experiments the effectors were separately added to crude retina extracts and incubated together with 26 mM glucose, 8 mM $MgCl_2$, 3.4 mM ATP, 13 mM Tris–HCl + 17 mM K_2HPO_4–KH_2PO_4 buffer at pH 7.4 for 1 hr at 37°C with or without added NAD^+. In a second set of experiments the effectors were separately preincubated with crude retina extracts for 1 hr (salicylate) or 4 hr (acetylsalicylate) at 0°C with or without added NAD^+.

These pretreated extracts were then incubated with glucose, Mg^{2+} and ATP for 1 hr at 37°C.

Na salicylate was assayed according to the colorimetric method described by Pankratz and Bandelin (1952).

RESULTS

1. *Anaerobic lactate formation from glucose in the presence of ATP and Mg^{2+}, in retina extracts after addition of salicylate, NAD^+* or both

Salicylate 31 mM strongly inhibits (−59%) anaero-

Table 1. Effect of 31 mM salicylate, 1.4 mM NAD^+ or both on the formation rate of glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-biphosphate (FDP), dihydroxyacetone phosphate (DAP), glyceraldehyde-3-phosphate (GAP) and lactate in bovine retina extracts anaerobically incubated with glucose, ATP and $MgCl_2$

Effector	G6P	F6P	FDP	DAP	GAP	Lactate
None	4.3	1.1	7.1	19.0	2.4	131
+ Salicylate	1.3	0.5	3.7	25.0	4.4	52
+ NAD^+	5.4	1.3	8.5	16.0	2.5	128
+ Salicylate + NAD^+	4.4	1.1	10.0	16.0	2.7	104

The metabolite levels are expressed in μmol/hr per g wet tissue.

bic lactate formation from glucose by retina extracts, in the presence of ATP and Mg^{2+} (Table 1). The addition of salicylate also results in lowered G6P (−70%), F6P (−60%) and FDP (−66%) levels, while DAP and GAP concentrations are increased (+36 and +83%, respectively) (Table 1). Similar but less pronounced effects are caused by lower salicylate concentrations (10 and 15 mM).

All the above effects are almost completely abolished by addition of 1.4 mM NAD^+ (Table 1).

2. *Anaerobic lactate formation from glucose in the presence of ATP and Mg^{2+}, in retina extracts preincubated for 1 hr at 0°C with salicylate, NAD^+ or both*

Anaerobic lactate formation from glucose in the presence of ATP and Mg^{2+}, is strongly reduced (−61%) in retina extracts preincubated for 1 hr at 0 C with 31 mM salicylate (Table 2). In the same incubation mixture G6P and F6P are greatly increased (+188%) while FDP, DAP and GAP levels are diminished (−94, −98 and −76%) (Table 2). Control experiments carried out by adding ATP to the preincubation mixture rather than adding it together with glucose and Mg^{2+} at the end of the preincubation time, produced identical results.

Preincubation of retina extracts with 3.5 mM NAD^+ together with 31 mM salicylate, followed by incubation with glucose, ATP and Mg^{2+} in anaerobic conditions, causes modifications in lactate formation rate and intermediate metabolite levels similar to those observed when retina extracts are preincubated with salicylate alone (Table 2). This result is not modified if ATP is present together with salicylate and NAD^+, during preincubation. NAD^+ concentrations up to 3.5 mM inhibit anaerobic glycolysis in bovine retina extracts (Rinaudo *et al.*, unpublished results).

3. *Effect of acetylsalicylate on anaerobic glycolysis in crude retina extracts*

Crude retina extracts incubated for 1 hr at 37°C with ATP, glucose, Mg^{2+} and 31 mM acetylsalicylate form 28% less lactate than controls incubated in the same conditions but without acetylsalicylate. Addition of 3.5 mM NAD^+ to the incubation mixture does not restore normal lactate production (Table 3). Thirty-one mM acetylsalicylate has however no effect on the concentrations of glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-biphosphate, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (Table 3).

Table 2. Anaerobic formation rate of glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-biphosphate (FDP), dihydroxyacetone phosphate (DAP), glyceraldehyde-3-phosphate (GAP) and lactate in bovine retina extracts preincubated for 1 hr at 0 C with 31 mM salicylate, 3.5 mM NAD or both

Effector	G6P	F6P	FDP	DAP	GAP	Lactate
None	4.0	0.9	8.9	12.0	2.5	93
+ Salicylate	11.5	2.5	0.5	0.9	0.6	36
+ NAD^+	4.1	1.4	9.2	10.0	2.1	83
+ Salicylate + NAD^+	10.2	2.4	0.5	0.8	0.4	40

The metabolite levels are expressed in μmol/hr per g wet tissue.

Table 3. Effect of 31 mM acetylsalate, 3.5 mM NAD or both in the formation rate of glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-biphosphate (FDP), dihydroxyacetone phosphate (DAP), glyceraldehyde-3-phosphate and lactate in bovine retina extracts anaerobically incubated with glucose, ATP and $MgCl_2$

Effector	G6P	F6P	FDP	DAP	GAP	Lactate
None	4.3	1.1	7.1	19.0	2.4	131
+ Acetylsalicylate	4.5	1.0	7.0	20.0	2.8	94
+ NAD^+	5.9	1.3	8.6	16.0	2.5	128
+ Acetylsalicylate + NAD^+	6.0	1.1	8.1	18.0	2.9	86

The metabolite levels are expressed in μmol/hr per g wet tissue.

Table 4. Anaerobic formation rate of glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-biphosphate (FDP), dihydroxyacetone phosphate (DAP), glyceraldehyde-3-phosphate (GAP) and lactate in bovine retina extracts preincubated for 4 hr at 0°C with 31 mM acetylsalicylate, 3.5 mM NAD^+ or both

Effector	G6P	F6P	FDP	DAP	GAP	Lactate
None	4.1	0.9	8.9	13.0	2.5	102
+ Acetylsalicylate	5.7	1.4	7.1	10.5	1.1	67
+ NAD^+	4.3	1.0	8.8	10.6	2.1	94
+ Acetylsalicylate + NAD^+	5.9	1.5	4.9	7.5	1.1	65

The metabolite levels are expressed in μmol/hr per g wet tissue.

4. *Effect of 4 hr of preincubation at 0°C with 31 mM acetylsalicylate*

Preincubation of crude retina extracts for 4 hr at 0°C with 31 mM acetylsalicylate results in a significant loss lactate production from glucose and ATP (−34.3%), with simultaneous accumulation of glucose-6-phosphate (+39%) and fructose-6-phosphate (+55%), and fall of fructose-1,6-biphosphate (−20%), dihydroxyacetone phosphate (−19%) and glyceraldehyde-3-phosphate (−56%). Addition of 3.5 mM NAD^+ to the preincubation mixture does not prevent these effects (Table 4).

One hour of preincubation with 31 mM acetylsalicylate causes a 28% decrease in lactate production but no significant changes in the intermediate metabolite profile.

DISCUSSION

Table 1 demonstrates an inhibition of anaerobic glycolysis in crude retina extracts of oxen, caused by addition of salicylate. The accumulation of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate suggests that salicylate, which inhibits yeast phosphoglycerate kinase (Larsson-Raznikiewicz & Wiksell, 1978), may inhibit the same enzyme in the retina, resulting in a slow down of the whole glycolytic flow. On the other hand the possibility of a direct inhibition of glyceraldehyde phosphate dehydrogenase cannot be ruled out. Since the accumulation of the triose phosphates is not followed by a correspondent increase in fructose-1,6-biphosphate, a strong inhibition of aldolase must be postulated. Moreover, a considerable inhibition of hexokinase and phosphofructokinase appears to be very likely, since both glucose-6-phosphate and fructose-1,6-biphosphate levels are significantly reduced after the addition of salicylate. These hypotheses are supported by previous observations on the effects of salicylate on crude extracts of brain, liver and kidney (Bargoni & Balocco, 1961) and smooth muscle (Sisini *et al.*, 1964), as well as on highly purified preparations of glyceraldehyde phosphate dehydrogenase (Bargoni, 1964).

Moreover it is very interesting to note that all the modifications induced by salicylate are almost completely abolished by addition of NAD^+ together with salicylate to the incubation mixture (Table 1). These results indirectly support the observations of Larsson-Raznikievicz & Wiksell (1978) and Einarsson *et al.* (1974), that the adenosine binding sites of NAD-linked dehydrogenases and ATP-dependent kinases are structurally similar, and that salicylate exerts a competitive action for the adenosine binding sites of both classes of enzymes.

The results of the experiments performed on extracts preincubated with salicylate alone or with salicylate and NAD^+ (Table 2), are difficult to correlate with the foregoing. If in fact preincubation with salicylate still results in an inhibition of the whole glycolytic flow, the levels of the intermediate metabolites substantially differ from those obtained by simultaneous incubation with salicylate. This suggests that glycolysis inhibition is achieved by different kinds in interactions between salicylate and glycolytic enzymes during the preincubation time. The accumulation of glucose-6-phosphate and fructose-6-phosphate together with the lowered levels of fructose-1,6-biphosphate and triose phosphates, that occurs as a consequence of preincubation, clearly indicates a specific inhibition of phosphofructokinase, rather than a combined action of salicylate on a number of enzymes as seems to be the case when preincubation is omitted. Moreover, neither anaerobic lactate formation nor normal levels of intermediary metabolites are restored in retina extracts by preincubation with 3.5 mM NAD^+ together with salicylate. In this instance phosphofructokinase inhibition would seem not to be competitive with NAD^+, in contrast to the results of the experiments performed without preincubation.

Salicylate therefore seems capable of inhibiting phosphofructokinase by two different mechanisms. The first involves the interaction of salicylate with the enzyme's adenosine binding site and results in an inhibition that is released by NAD^+. To explain the second we postulate the existence of an additional site on the enzyme, insensitive of NAD^+, that is highly specific but has a low affinity for salicylate.

Tables 3 and 4 demonstrate an inhibition of anaerobic lactate formation by acetylsalicylate in bovine retina extracts either preincubated or directly incubated with the effector. However acetylsalicylate modifies the intermediate metabolite levels only in preincubated extracts. Glucose-6-phosphate and fructose-6-phosphate do accumulate, fructose-1,6-biphosphate, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate fall down. These results suggest a distinct inhibition of phosphofructokinase. On the other hand a concomitant inhibition of one or more enzymes catalyzing some of the following steps in the pathway may also be possible: a decrease of aldolase activity could be involved in the fall of dihydroxyace-

tone phosphate and glyceraldehyde-3-phosphate, while a decrease in lactate dehydrogenase activity would contribute to the slow down on the lactate production. In any case, the inhibition of the anaerobic glycolysis seems to occur rapidly and it is not competitive with NAD^+.

Moreover we obtained identical results preincubating crude retina extracts with salicylate. This suggests that functional groups common to both compounds may establish similar interactions with the same enzymes. However, when salicylate is added directly to the incubation mixture (omitting preincubation) a more complex response is elicited, which is also uniquely removed by NAD^+. Salicylate appears then capable of a dual action on anaerobic glycolysis, depending on the experimental condition, only one of which is shared by acetylsalicylate.

It could also be speculated that acetylsalicylate, which is known to acetylate several proteins (Flower *et al.*, 1980; Pinckard *et al.*, 1970), and enzymes (Han *et al.*, 1978; Roth & Siok, 1978; Siegel *et al.*, 1980a,b; Van Der Ouderaa *et al.*, 1980) may produce its effects on glycolysis by acetylating same critical aminoacid residues of several enzymes. But we believe that this phenomenon can be excluded, as in our experiments we have never been able to demonstrate a formation of salicylate from acetylsalicylate even when bovine retina extracts were preincubated for 4 hr at 0°C with the inhibitor.

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