Metabolic effects of bacitracin in isolated rat hepatocytes

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Bacitracin is a proteolytic inhibitor which interacts with the intracellular processing of insulin. Its effects on pyruvate, fatty acid and amino acid metabolism were examined in rat hepatocyte suspensions. Bacitracin (0.25–1.0 mM) increased the oxidation of [1-¹⁴C]pyruvate by 50–70% and presumably therefore increased the flux through pyruvate dehydrogenase. This was found both in the presence of extracellular Ca²⁺ and in its absence, but not in the presence of 2 mM-2-chloropropionate, which inhibits pyruvate dehydrogenase kinase. Insulin did not further stimulate [1-¹⁴C]pyruvate oxidation in the presence of 1 mM-bacitracin. Bacitracin decreased ¹⁴CO₂ formation from [2-¹⁴C]pyruvate (20–40%) and [U-¹⁴C]palmitate (30–70%), suggesting a decreased flux through the tricarboxylic acid cycle. Fatty acid oxidation before acetyl-CoA formation was also decreased. Bacitracin decreased the incorporation of label from [³H]leucine into protein in the absence of insulin, but not in its presence. Bacitracin is commonly used in studies on insulin action. Our results suggest that in such studies the effects noted may be related not only to an interaction of bacitracin with the intracellular processing of insulin but also to direct metabolic effects of bacitracin independent of insulin.

Bacitracin, a cyclic polypeptide derived from *Bacillus licheniformis*, is widely used as an antibiotic and as a proteolytic inhibitor to inhibit the degradation of insulin and glucagon in studies on hormone–receptor binding and action (Makinen, 1972; Desbuquois *et al.*, 1974; Carpentier *et al.*, 1979; Dolais-Kitabgi *et al.*, 1981). It has been used primarily to inhibit the extracellular degradation of polypeptide hormones in studies with isolated plasma membranes and intact hepatocyte and adipocyte preparations. However, it has more recently been shown to affect the intracellular processing of insulin, in studies with freshly isolated hepatocytes (Juul & Jones, 1982) and cultured foetal hepatocytes (Plas & Desbuquois, 1982).

Studies with isolated rat adipocytes (Larner *et al.*, 1982) showed that, at concentrations up to 5 mM, bacitracin prevented insulin degradation, but did not block insulin action. The latter was blocked at 9 mM-bacitracin. In rat adipocytes, bacitracin inhibited cyclic AMP-induced lipolysis, and it was suggested that bacitracin can be used to study lipolysis in the absence of other insulin-like effects (Heckemeyer *et al.*, 1982). There have, however, been few studies on the metabolic effects of

bacitracin. In view of its wide usage at high concentrations (0.5–2 mM) in studies on insulin binding and action, we have examined some of the metabolic effects of bacitracin on rat hepatocyte suspensions at concentrations currently used in insulin studies.

Experimental

Chemicals

Bacitracin (zinc-free), collagenase (type I), bovine serum albumin (fraction V), substrates and co-factors were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other enzymes were from Boehringer (Mannheim, Germany). Crystalline pig insulin was a gift from Novo Laboratories (Copenhagen, Denmark). The radiochemicals sodium [1-¹⁴C]pyruvate, sodium [2-¹⁴C]pyruvate, [U-¹⁴C]palmitic acid, NaH¹⁴CO₃ and L-[4,5-³H]leucine were from Amersham International (Amersham, Bucks., U.K.). Minimum Essential Medium (Eagle) amino acids were from Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.). 2-Chloropropionate was from Kodak (Kirkby, Liverpool, U.K.). All other chemicals were from BDH Chemicals (Poole,

Dorset, U.K.). The bovine serum albumin was defatted and dialysed as described by Chen (1967). The [^{14}C]pyruvate and [$2\text{-}^{14}\text{C}$]pyruvate were purified before use by cellulose t.l.c. with diethyl ether/formic acid/water, (7:2:1, by vol.) as developing solvent, and eluted from the cellulose in 5 mM-sodium pyruvate. After purification, the acid-volatile radioactivity in blank incubations was less than 0.13%.

Rats

Male rats (body wt. 180–220 g) of the Albino Wistar strain were used. They were bred in the Animal House of the University of Newcastle Medical School and fed on standard laboratory chow *ad libitum*.

Hepatocyte preparation

Parenchymal hepatocyte suspensions were prepared between 09:00 and 11:00 h by collagenase perfusion of the liver (Seglen, 1976). The incubation medium was a modified Krebs–Henseleit (1932) buffer containing 10 mM- NaHCO_3 , 8 mM-glucose and 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonate], pH 7.4 at 37°C, with or without 2% (w/v) bovine serum albumin. The buffer was gassed with O_2/CO_2 (19:1). After dissociation of the liver cells and filtration through a 60 μm mesh, the cells were preincubated in the albumin-free medium for 25 min, washed twice in the same medium and once in the albumin-containing medium. They were then resuspended in the albumin buffer. All centrifugations were at 50 g for 2 min. For studies on the effects of extracellular Ca^{2+} , the cells were washed in Ca^{2+} -free medium and divided into batches to which were added CaCl_2 or neutralized EGTA solution, final concns. 2.5 and 1.0 mM respectively. The cell concentration was determined with a haemocytometer, and cell viability was estimated by Trypan Blue (0.2%, w/v) exclusion. Cell viability usually exceeded 90%.

Incubation conditions

For the determination of rates of $^{14}\text{CO}_2$ formation from ^{14}C -labelled substrates or $^{14}\text{CO}_2$ fixation into non-volatile metabolites, the cell suspension was incubated in a final volume of 1.0 ml of albumin-containing medium in 25 ml glass Erlenmeyer flasks containing a Microfuge tube. The flasks containing all additions other than the labelled substrate were gassed with O_2/CO_2 (19:1), stoppered and equilibrated for 10 min in a shaking water bath at 37°C. The reaction was started by the addition of substrate and stopped with 200 μl of 2 M- HClO_4 . Hyamine hydroxide (10-X) [250 μl of a 10% (w/v) soln. in methanol] was injected into the Microfuge tube for absorption of $^{14}\text{CO}_2$. The flasks were left on ice for 3 h. The Hyamine was transferred to 10 ml of scintillation cocktail (Scintran; BDH) and the

radioactivity determined. For the determination of acid-soluble radioactivity ([^{14}C]palmitate and $\text{H}^{14}\text{CO}_3^-$ incubations) the flask contents were centrifuged at 3000 rev./min (1000 g) for 15 min at 4°C and the radioactivity was determined in duplicate 300 μl samples. Incorporation of L-[4,5- ^3H]leucine into protein was determined in a final volume of 350 μl of medium supplemented with Minimum Essential Medium (Eagle) amino acids and 2 mM-glutamine in 11 ml polypropylene tubes. The final L-leucine concentration was 0.4 mM. The protein was precipitated with trichloroacetic acid and treated as described by Clark & Hansen (1980). Adenine nucleotides and ketone bodies were assayed in the neutralized HClO_4 extracts by enzymic methods (ADP and AMP, Adam, 1963; ATP, Lamprecht & Trautschold, 1963; acetoacetate and D-3-hydroxybutyrate, Williamson *et al.*, 1962). The following final cell concentrations were used for the various incubations: [^{14}C]pyruvate, 0.2×10^6 – 0.4×10^6 cells/ml; [^{14}C]palmitate, 0.6×10^6 – 0.9×10^6 cells/ml; $\text{H}^{14}\text{CO}_3^-$ and L-[4,5- ^3H]leucine, 1.3×10^6 – 3.5×10^6 cells/ml; and metabolite determinations 3.5×10^6 – 4.0×10^6 cells/ml.

Incubations were performed in triplicate at one or two time points (pyruvate, 8 min; palmitate, 40 min; $\text{H}^{14}\text{CO}_3^-$, 30 min; protein synthesis, 90 min; metabolites, 60 and 100 min). The time courses were linear for at least 20 min for 0.5 mM-pyruvate oxidation, 60 min for 1 mM-palmitate oxidation and $^{14}\text{CO}_2$ fixation and 180 min for [^3H]leucine. Rates are expressed per g wet wt. of hepatocytes. The viable hepatocyte number per g wet wt. of hepatocytes was between 111 and 118 million cells. Except where stated otherwise, results are means $\pm$ S.E.M. for a given number of hepatocyte preparations. Statistics was by Student's paired *t* test.

Results and discussion

Effects of bacitracin on the oxidation of [$1\text{-}^{14}\text{C}$]pyruvate

The formation of $^{14}\text{CO}_2$ from [$1\text{-}^{14}\text{C}$]pyruvate in intact hepatocytes is a measure of the flux through pyruvate dehydrogenase. Only a small percentage (<5%) of the $^{14}\text{CO}_2$ is due to the activity of the tricarboxylic acid cycle, whose C_4 intermediates become labelled by pyruvate carboxylation (Stucki & Walter, 1972).

In the present study, bacitracin (0.25–1.0 mM) increased the formation of $^{14}\text{CO}_2$ from [$1\text{-}^{14}\text{C}$]pyruvate by 50–70% (Table 1). Bacitracin has been suggested to increase the transport of bivalent ions (Heckemeyer *et al.*, 1982). An increased uptake of extracellular Ca^{2+} would be expected to lead to influx of Ca^{2+} into the mitochondria and activation of pyruvate dehydrogenase by stimulation of the Ca^{2+} -sensitive phosphatase (Denton *et al.*, 1972).

Table 1. *Effects of bacitracin on ¹⁴CO₂ formation from [1-¹⁴C]- and [2-¹⁴C]-pyruvate by rat hepatocyte suspensions* Hepatocytes were incubated with 0.5 mM-[1-¹⁴C]- or [2-¹⁴C]-pyruvate (0.04 μCi/μmol) as described in the Experimental section. Rates are expressed as μmol of ¹⁴CO₂ formed/h per g wet wt. of hepatocytes. Results are means ± S.E.M. for the numbers of experiments shown in parentheses. Values that are significantly different from the controls with no bacitracin are shown: **P* < 0.05; ***P* < 0.005.

Bacitracin (mM)	¹⁴ CO ₂ formed (μmol/h per g wet wt.)		
	[1- ¹⁴ C]Pyruvate + 2.5 mM-Ca ²⁺ (5)	[1- ¹⁴ C]Pyruvate + 1 mM-EGTA (3)	[2- ¹⁴ C]Pyruvate + 2.5 mM-Ca ²⁺ (4)
0	51.6 ± 4.9	54.5 ± 6.7	10.2 ± 0.3
0.25	77.6 ± 8.9*	74.2 ± 16.3*	8.4 ± 0.8*
0.50	79.9 ± 6.7**	79.3 ± 11.8*	6.8 ± 0.5**
1.0	86.7 ± 4.1**	87.3 ± 1.4*	6.3 ± 0.7**

Fig. 1. *Effects of bacitracin and insulin on the oxidation of [1-¹⁴C] pyruvate by rat hepatocyte suspensions* Hepatocytes were incubated with 0.5 mM-[1-¹⁴C]-pyruvate with or without bacitracin (1.0 mM) as indicated: □, no added insulin; ▨, 0.85 nM-insulin; ■, 8.5 nM-insulin. The rate of formation of ¹⁴CO₂ from [1-¹⁴C]pyruvate is expressed as μmol of pyruvate transformed/h per g wet wt. of hepatocytes. Results are means ± S.E.M. for triplicate incubations in a representative experiment.

Bacitracin increased the oxidation of [1-¹⁴C]pyruvate both in the presence of extracellular Ca²⁺ and in Ca²⁺-free medium containing the chelating agent EGTA (Table 1), suggesting that the stimulation of [1-¹⁴C]pyruvate oxidation by bacitracin may not be the result of an increased uptake of extracellular Ca²⁺. It does not, however, exclude the possibility of an interaction of bacitracin with the Ca²⁺-extrusion process.

In the absence of bacitracin, insulin (0.85–8.5 nM) increased (*P* < 0.05) the oxidation of [1-¹⁴C]pyruvate by 12–16% (Fig. 1). In the presence of 1.0 mM-bacitracin, insulin did not further stimulate the oxidation of [1-¹⁴C]pyruvate (Fig. 1).

Fig. 2. *Effects of bacitracin and 2-chloropropionate on the oxidation of [1-¹⁴C]pyruvate by rat hepatocyte suspensions*

Hepatocytes were incubated with 0.5 mM-pyruvate with or without 2-chloropropionate (2 mM) as indicated: □, no added bacitracin; ▨, 1.0 mM-bacitracin. The rate of formation of ¹⁴CO₂ from [1-¹⁴C]pyruvate is expressed as μmol of pyruvate transformed/h per g wet wt. of hepatocytes. Results are means ± S.E.M. for triplicate incubations in a representative experiment.

2-Chloropropionate (2 mM) increased (*P* < 0.005) the oxidation of [1-¹⁴C]pyruvate by 240% (Fig. 2). 2-Chloropropionate stimulates pyruvate dehydrogenase by inhibiting pyruvate dehydrogenase kinase (Whitehouse *et al.*, 1974). The stimulatory effects of bacitracin and 2-chloropropionate on [1-¹⁴C]pyruvate oxidation were not additive (Fig. 2). This might suggest that bacitracin could be exerting an effect on the kinase.

Effects of bacitracin on the oxidation of [2-¹⁴C]-pyruvate

Bacitracin (0.25–1.0 mM) decreased the oxidation of [2-¹⁴C]pyruvate by 19–39% (Table 1), and

Table 2. *Effects of bacitracin on the conversion of [U-¹⁴C]palmitate into ¹⁴CO₂ and ¹⁴C-labelled acid-soluble metabolites by rat hepatocyte suspensions*

Hepatocytes were incubated with 1 mM-[U-¹⁴C]palmitate (0.3 μ Ci/ μ mol) as described in the Experimental section. The rates of conversion of [U-¹⁴C]palmitate into ¹⁴CO₂ and ¹⁴C-labelled acid-soluble metabolites are expressed as μ mol of palmitate transformed/h per g wet wt. of hepatocytes. Results are means $\pm$ S.E.M. for $n = 5$. Values that are significantly different from the controls with no bacitracin are shown: * $P < 0.05$; ** $P < 0.005$.

Bacitracin (mM)	[U- ¹⁴ C]Palmitate $\rightarrow$ ¹⁴ CO ₂		[U- ¹⁴ C]Palmitate $\rightarrow$ ¹⁴ C-labelled metabolites	
	(μ mol of palmitate/h per g wet wt.)	(% of control)	(μ mol of palmitate/h per g wet wt.)	(% of control)
0	0.93 $\pm$ 0.09	100	21.2 $\pm$ 1.1	100
0.25	0.69 $\pm$ 0.14*	71.0 $\pm$ 7.7	19.9 $\pm$ 1.5*	93.4 $\pm$ 2.2
0.5	0.57 $\pm$ 0.14**	57.3 $\pm$ 9.3	19.0 $\pm$ 1.6*	88.9 $\pm$ 2.9
1.0	0.34 $\pm$ 0.13**	32.3 $\pm$ 10.6	16.4 $\pm$ 2.3*	75.9 $\pm$ 6.8

presumably, therefore, the flux through the tricarboxylic acid cycle (Stucki & Walter, 1972). Thus the increased flux through pyruvate dehydrogenase was not associated with a parallel increase in the complete oxidation of pyruvate. This finding is analogous to the effect of dichloroacetate, which activates pyruvate dehydrogenase (Whitehouse *et al.*, 1974) and increases the flux through the enzyme in intact hepatocytes (Agius & Vaartjes, 1982) but it decreases the oxidation of [2-¹⁴C]-pyruvate (L. Agius & W. J. Vaartjes, unpublished work). Bacitracin may have a direct effect on the tricarboxylic acid cycle, or alternatively the increased production of ATP through the pyruvate dehydrogenase reaction may result in decreased rates of other mitochondrial oxidative reactions.

Effects of bacitracin on the oxidation of [U-¹⁴C]-palmitate

To investigate whether bacitracin affects other mitochondrial oxidation reactions, the conversion of [U-¹⁴C]palmitate into ¹⁴CO₂ and ¹⁴C-labelled acid-soluble metabolites was examined. In the absence of bacitracin, the conversion of palmitate into ¹⁴CO₂ was less than 5% of its conversion into acid-soluble metabolites (mainly acetoacetate and D-3-hydroxybutyrate). Preliminary experiments showed that when D-3-hydroxybutyrate was converted into acetoacetate, and the acetoacetate precipitated as the Denige's salt (McGarry *et al.*, 1970), the radioactivity in the acetoacetate accounted for more than 90% of the radioactivity in the neutralized acid-soluble supernatant, corrected for blank incubations. In the presence of bacitracin (0.25–1.0 mM), incorporation of label from [U-¹⁴C]palmitate into CO₂ and acid-soluble metabolites was decreased (Table 2), suggesting decreased β -oxidation of fatty acids. The inhibition of ¹⁴CO₂ formation (29–68%) was greater ($P < 0.01$) than the inhibition of label incorporation into acid-soluble metabolites (7–24%),

suggesting selective inhibition of the oxidation of acetyl-CoA to CO₂ via the tricarboxylic acid cycle. There is evidence that the oxidation of acetyl-CoA to CO₂ may be more tightly linked to hepatic ATP requirements than is fatty acid β -oxidation (Berry *et al.*, 1983). The increased ATP availability via the pyruvate dehydrogenase reaction may account for the inhibition of ¹⁴CO₂ formation from [U-¹⁴C]-palmitate and [2-¹⁴C]pyruvate.

¹⁴CO₂ reincorporation by exchange reactions

Metabolic rates expressed as the formation of ¹⁴CO₂ from ¹⁴C-labelled substrates may represent an underestimate of the oxidation of substrate, because the ¹⁴CO₂ generated can be reincorporated into intermediary metabolites via exchange reactions (Tomera *et al.*, 1982). In the present study, the effects of bacitracin on the incorporation of ¹⁴CO₂ into acid-soluble metabolites was examined in order to establish whether the observed effects of bacitracin on the accumulation of ¹⁴CO₂ from ¹⁴C-labelled substrates result from changes in substrate oxidation or CO₂ reincorporation.

The hepatocytes were incubated with H¹⁴CO₃⁻, assuming that HCO₃⁻ and CO₂ equilibrate as a single pool. After termination of the incubation with HClO₄, the liberated CO₂ was absorbed in Hyamine. Recovery of radioactivity in the Hyamine fraction and acid-soluble (non-volatile metabolites) supernatant was >95%. The radioactivity in the pellet (HClO₄-insoluble) was negligible. The radioactivity incorporated into metabolites non-volatile in acid was linear with time during a 60 min time course. The percentage of HCO₃⁻ plus CO₂ from the medium that was incorporated into acid-soluble metabolites by a cell concentration of 1.3×10^6 cells/ml was 1.3% at 20 min, 3.2% at 40 min and 4.5% at 60 min. The total rate of ¹⁴CO₂ fixation calculated from the radioactivity in the non-volatile acid-soluble fraction and the initial specific radioactivity of the

Table 3. *Effects of bacitracin on the incorporation of $^{14}\text{CO}_2$ into acid-soluble non-volatile metabolites by rat hepatocyte suspensions*

Hepatocytes were incubated in buffer containing $10\text{ mM-H}^{14}\text{CO}_3^-$ ($0.25\text{ }\mu\text{Ci}$ in a final volume of 1 ml) as described in the Experimental section. Rates of incorporation of $^{14}\text{CO}_2$ into metabolites non-volatile in acid are expressed as: (1) μmol of $^{14}\text{CO}_2$ incorporated/h per g wet wt. of hepatocytes; (2) the percentage of the HCO_3^- plus CO_2 in the medium incorporated/h per 10^6 cells. Results are means $\pm$ S.E.M. for $n=4$. Values that are significantly different from the controls with no bacitracin are shown: $*P<0.05$.

	(1) $^{14}\text{CO}_2$ incor- poration ($\mu\text{mol/h}$ per g wet wt.)	(2) Percentage of $\text{HCO}_3^- + \text{CO}_2$ in medium incorporated/h per 10^6 cells
Bacitracin (mM)		
0	42.5 ± 2.9	3.60 ± 0.25
0.25	43.8 ± 2.2	3.71 ± 0.18
0.5	$45.1 \pm 1.8^*$	3.82 ± 0.15
1.0	$47.3 \pm 1.3^*$	4.01 ± 0.11

HCO_3^- plus CO_2 was $43.7 \pm 5.6\text{ }\mu\text{mol/h}$ per g wet wt. of hepatocytes (mean $\pm$ S.E.M., $n=7$). This assumes that the total concentration and specific radioactivity of HCO_3^- plus CO_2 remained constant during the incubation and that the extracellular CO_2 equilibrates with the CO_2 in the cytosolic and mitochondrial compartments. The linearity of the time course and the low percentage of total label ($<5\%$) incorporated into non-volatile compounds support the first assumption. The validity of the assumption that extracellular and intracellular CO_2 equilibrate (Tomera *et al.*, 1982) remains to be verified. Bacitracin ($0.5\text{--}1.0\text{ mM}$) resulted in a small but significant increase ($6\text{--}11\%$) in the incorporation of $^{14}\text{CO}_2$ into non-volatile acid-soluble metabolites (Table 3). The finding that less than 5% of the HCO_3^- plus CO_2 in the medium was incorporated into acid-soluble metabolites/h per 10^6 cells (Table 3) implies that if the $^{14}\text{CO}_2$ formed from the oxidation of labelled substrates equilibrates with the extracellular CO_2 , then the estimated rates of $^{14}\text{CO}_2$ formation approach the true rates of substrate oxidation to within 95%. Bacitracin increased $^{14}\text{CO}_2$ fixation from 3.6 to 4.0%. Since bacitracin inhibited $^{14}\text{CO}_2$ formation from $[2\text{-}^{14}\text{C}]\text{-pyruvate}$ by 20–40% and from $[\text{U-}^{14}\text{C}]\text{palmitate}$ by 30–70%, it is unlikely that CO_2 fixation contributed significantly to the estimated decrease in CO_2 formation from these substrates.

Cellular adenine nucleotides and ketone-body concentrations

When hepatocytes were incubated with bacitracin ($0.25\text{--}1.0\text{ mM}$) for 60 or 100 min the cell

contents of ATP, ADP and AMP were unchanged [controls: ATP, 2.38 ± 0.08 ; ADP, 0.87 ± 0.08 ; AMP, 0.19 ± 0.02 ; bacitracin ($0.25\text{--}1\text{ mM}$): ATP, 2.24 ± 0.18 ; ADP, 0.93 ± 0.06 ; AMP, 0.19 ± 0.04 ($\mu\text{mol/g}$ wet wt.; means $\pm$ S.E.M. for $n=3$)]. This suggests that there was no leakage of adenine nucleotides from the cell and is consistent with the observation that the metabolic effects of bacitracin on adipocytes were reversible on removal of bacitracin (Heckemeyer *et al.*, 1982), implying that it did not cause permanent cell damage.

In hepatocytes from fed rats incubated with 0.3 mM-palmitate , bacitracin decreased the $[3\text{-hydroxybutyrate}]/[\text{acetoacetate}]$ ratio (control, 0.73 ± 0.04 ; $0.25\text{ mM-bacitracin}$, 0.61 ± 0.02 ; $0.50\text{ mM-bacitracin}$, 0.60 ± 0.04 ; 1.0 mM-bacitracin , 0.58 ± 0.06 ; means $\pm$ S.E.M. for $n=3$) suggesting a more oxidized mitochondrial NADH/NAD $^+$ redox state. This was not associated with a decrease in ketone-body formation [control, 34.8 ± 7.7 ; $0.25\text{ mM-bacitracin}$, 38.7 ± 10.9 ; $0.50\text{ mM-bacitracin}$, 40.7 ± 6.4 ; 1.0 mM-bacitracin , 44.6 ± 6.4 (μmol of ketone bodies formed/h per g wet wt. of hepatocytes)]. However, a decrease in ketone-body formation with bacitracin was observed with hepatocytes from 24 h-starved rats. The latter may be due to inhibition of fatty acid oxidation (Table 2), whereas the failure to observe a decrease in ketone-body formation in hepatocytes from fed rats may be the result of increased acetyl-CoA formation via pyruvate dehydrogenase. An increase in ketone-body formation from pyruvate is also observed with dichloroacetate (Agius & Vaartjes, 1982), which activates pyruvate dehydrogenase (Whitehouse *et al.*, 1974).

Effects of bacitracin and insulin on $[^3\text{H}]\text{leucine}$ incorporation into protein

When hepatocytes were incubated with $\text{L-[4,5-}^3\text{H]leucine}$, in the absence of insulin, bacitracin decreased the incorporation of label into protein (Fig. 3). Insulin had no effect on the incorporation of label in the absence of bacitracin. In the combined presence of bacitracin and insulin the incorporation was higher than in the presence of insulin alone (Fig. 3). In the present study, the final L-leucine concentration was 0.4 mM . Using higher concentrations of leucine ($10\text{--}20\text{ mM}$), Clark & Hansen (1980) showed that insulin increased $[^3\text{H}]\text{leucine}$ incorporation into protein. It was assumed that the intracellular and extracellular specific radioactivities of leucine were equal and that the effect of insulin was on protein synthesis. In the present study the incorporation of ^3H label into protein is expressed as the rate of leucine incorporation into protein, assuming that the leucyl-tRNA specific radioactivity is equal to the specific radioactivity of the extracellular leucine (Khairallah & Mortimore,

Fig. 3. Effects of bacitracin and insulin on the incorporation of [^3H]leucine into protein by rat hepatocyte suspensions

Hepatocytes were incubated with Minimal Essential Medium amino acids and L-[4,5- ^3H]leucine ($1\ \mu\text{Ci}/\mu\text{mol}$) as described in the Experimental section. The incubations were either in the absence (○) or in the presence (●) of insulin ($85\ \text{nM}$). Results are means $\pm$ S.E.M. for $n = 3$. The rate of incorporation of [^3H]leucine into protein is expressed as nmol of leucine incorporated/h per 10^6 cells. Values that are significantly different from the controls with no added bacitracin are shown: * $P < 0.01$.

1976). It should be emphasized, however, that a change in label incorporation could arise from a change in specific radioactivity of the intracellular ^3H -labelled leucine or leucyl-tRNA rather than a change in protein synthesis. Thus the increased incorporation of label in the combined presence of insulin and bacitracin may result from increased protein synthesis and/or an increased intracellular specific radioactivity because of inhibition of protein degradation or increased amino acid uptake.

Conclusions

Metabolic effects of bacitracin. Bacitracin inhibits cyclic AMP-induced lipolysis in rat adipocytes (Heckemeyer *et al.*, 1982), and it increases glucose incorporation into glycogen in cultured foetal hepatocytes (Plas & Desbuquois, 1982). The present study shows that bacitracin, at concentrations currently used to inhibit hormone degradation, increases the flux through pyruvate dehydrogenase but decreases tricarboxylic acid-cycle activity. These changes were associated with a more oxidized mitochondrial redox state, but no change in total cellular adenine nucleotides. It is unlikely that these effects of bacitracin are secondary to an increase in plasma-membrane permeability to Ca^{2+} (Heckemeyer *et al.*, 1982), since an increased oxidation of

[1- ^{14}C]pyruvate was observed also in the absence of extracellular Ca^{2+} . However, other ionic changes cannot be excluded. A key question is what is the primary site(s) of action of bacitracin. The more oxidized mitochondrial redox state may account for the activation of pyruvate dehydrogenase by decreased end-product inhibition and inactivation of the kinase which converts the enzyme into its inactive phosphorylated form.

Effects on insulin action. In the present study, the stimulatory effect of bacitracin on [1- ^{14}C]pyruvate oxidation was larger than that of insulin, and in the presence of bacitracin insulin caused no further stimulation. In cultured hepatocytes (Plas & Desbuquois, 1982), the effect of insulin on [^{14}C]glucose incorporation into glycogen was decreased in the presence of 2 mM-bacitracin. Higher concentrations of bacitracin (9 mM) were necessary to block the action of insulin on glucose oxidation in rat adipocytes (Larner *et al.*, 1982). Effects of bacitracin on insulin action have been interpreted in terms of: (i) inhibition of extracellular degradation of hormone and increased binding (Desbuquois *et al.*, 1974); (ii) an interaction of bacitracin with the intracellular degradation of insulin (Juul & Jones, 1982; Plas & Desbuquois, 1982); (iii) the post-receptor events in insulin action may involve the generation of a low-molecular-weight peptide, and bacitracin may inhibit the formation of the peptide (Larner *et al.*, 1982). Since bacitracin exerts marked effects (in the absence of added hormones) on the metabolism of pyruvate and fatty acids at concentrations below 1 mM, the block of insulin action at millimolar bacitracin concentrations may be related to an effect of bacitracin at the level of the metabolic pathway rather than an interaction with the intracellular processing of insulin. Although bacitracin suppressed the effects of insulin on [1- ^{14}C]pyruvate oxidation in the present study and glucose incorporation into glycogen in cultured hepatocytes (Plas & Desbuquois, 1982), it increased the incorporation of [^3H]leucine into protein in the presence of insulin, but not in its absence. This might suggest an intracellular interaction of bacitracin and insulin. Whatever the mechanism of action, the metabolic effects of bacitracin need also to be considered in studies involving bacitracin and insulin action.

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