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Metabolic Interactions of Glucose, Acetoacetate and Adrenaline in Rat Submaxillary Gland *in vitro*

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1. The metabolic interactions between glucose, acetoacetate and adrenaline were studied in submaxillary-gland slices. 2. Acetoacetate (2.5mM) inhibited glucose removal by 22% and entry of glucose carbon into the tricarboxylic acid cycle by 54%. 3. Acetoacetate caused an increase in [glucose 6-phosphate] together with an increase in [citrate], a finding that suggests that the phosphofructokinase step might be inhibited by the elevated [citrate]. Support for this suggestion was obtained in experiments in which fluoroacetate was used to elevate [citrate]. 4. A further site of action of acetoacetate at the pyruvate dehydrogenase step was suggested by an increase in the lactate+pyruvate pool, and the finding that pyruvate removal and [3-^{14}C]pyruvate oxidation were inhibited by acetoacetate. 5. Adrenaline, a stimulator of secretion by this tissue, increased glucose removal by 25%. Adrenaline increased glucose removal to the same extent when acetoacetate was also present in the incubation medium. In both cases the increase was accompanied by a fall in [glucose 6-phosphate]. 6. Adrenaline also overcame the inhibition of pyruvate removal caused by acetoacetate. 7. The tissue [ATP] decreased by about 50% on addition of adrenaline, and a similar fall was observed *in vivo* after adrenergic stimulation by isoproterenol. 8. Omission of Ca^{2+} from the medium prevented the fall in [glucose 6-phosphate] and [ATP] caused by adrenaline, although adrenaline was still able to stimulate glucose removal. The inhibitory effect of acetoacetate on glucose removal was reversed by adrenaline, but there was no stimulation above the control rates. Inhibition of pyruvate removal by acetoacetate was not overcome by adrenaline in the absence of Ca^{2+}. 9. Dibutyryl cyclic AMP had no effect on glucose removal or on [ATP]. 10. Possible mechanisms by which adrenaline can bring about its metabolic effects are discussed.

Ketone bodies (acetoacetate and 3-hydroxybutyrate) and fatty acids can decrease glucose uptake by cardiac and diaphragm muscle (Williamson & Krebs, 1961; Randle *et al.*, 1964). This preferential utilization of ketone bodies and fatty acids compared with glucose involves regulatory interactions between the various metabolic pathways at the sites of glucose transport, hexokinase, phosphofructokinase and pyruvate dehydrogenase (Randle *et al.*, 1964). It is of interest whether these metabolic interactions are common to all tissues capable of utilizing the alternative substrates and whether they can be directly influenced by hormones or by changes in tissue function. In heart, high concentrations of insulin can overcome the block in transport of glucose, but not the inhibition of its intracellular metabolism brought about by ketone bodies and fatty acids. Similarly, there is no evidence that an increase in ventricular work can affect the competition between glucose and palmitate as substrates for oxidative metabolism (for reviews, see Opie, 1968; Neely & Morgan, 1974).

The rat submaxillary gland was chosen for the present experiments because it has relatively high activities of the enzymes of ketone-body utilization (Williamson *et al.*, 1971) and is known to oxidize ketone bodies (Ueha *et al.*, 1971) and palmitate (Pritchard, 1972) as well as glucose. Another feature which makes salivary gland an appropriate tissue is that it can be used for studying the relationships between metabolism and function (e.g. secretion) (Deutsch & Raper, 1936, 1938; Babad *et al.*, 1967; Feinstein & Schramm, 1970; Ueha *et al.*, 1971).

The object of the present work was to investigate the effects of acetoacetate on glucose metabolism by the gland and to test if adrenaline, a secretagogue in this tissue, could modify possible metabolic interactions.

The results indicate that acetoacetate inhibits glucose uptake by submaxillary-gland slices, and that

adrenaline stimulates glucose utilization and abolishes the inhibitory effect of acetoacetate. Possible mechanisms for these effects of adrenaline are discussed.

Experimental

Materials

Male rats of the Wistar strain, weighing 150–200g with free access to food were used for all experiments.

Enzymes, coenzymes, 6-*N*,2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate and sodium pyruvate were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Adrenaline for injection (0.1%, w/v; 1ml ampoule) was obtained from Macarthys Ltd., Romford, Essex, U.K. DL-Isoproterenol hydrochloride was obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. [6-^{14}C]Glucose and [3-^{14}C]pyruvate were from The Radiochemical Centre Ltd., Amersham, Bucks., U.K. Hydroxide of Hyamine 10-X, butyl-PBD[5-(4-biphenylyl)-2-(4-t-butylphenyl-1-oxa-3,4-diazole] and naphthalene were obtained from Packard Instrument Ltd., Caversham, Berks., U.K. Sodium fluoroacetate was a gift from Dr. B. C. Saunders, Cambridge. Glucose, ethyl acetoacetate and all other chemicals were from BDH Chemicals Ltd., Poole, Dorset, U.K. Sodium acetoacetate was prepared from ethyl acetoacetate as described by Krebs & Eggleston (1945).

Methods

Experiments in vitro. The rats were killed by cervical dislocation and the submaxillary glands dissected out into ice-cold saline (Krebs & Henseleit, 1932). The glands were sliced longitudinally from base to apex with a razor-blade and the slices (approx 0.2–0.3mm thick) placed in the ice-cold saline and used for the experiments within 30min.

Incubations were carried out for 1h at 37°C in 50ml Erlenmeyer flasks with Krebs–Henseleit (1932) bicarbonate saline (5ml) containing the appropriate additions: glucose (5mM), acetoacetate (2.5mM), pyruvate (2mM) and adrenaline (10μg/ml), gassed with O_2+CO_2 (95:5) and sealed with rubber stoppers. All variations in the incubations were performed in duplicate. Each flask contained tissue (200±10mg wet wt.) chosen randomly from the slices prepared from up to 12 glands (six rats) for each experiment.

At the end of the incubation period the slices were quickly removed from the medium, blotted on Whatman no. 1 filter paper and homogenized in 6% (w/v) $HClO_4$ (5ml) with a motorized homogenizer. The extracts were centrifuged for 30min at 30000*g* in the cold. The medium was acidified with 30% $HClO_4$ (0.5ml) and centrifuged for 10min at 2000*g* in the cold. All the supernatants were neutralized with 20% (w/v) KOH and left in ice–water for 10min to allow the $KClO_4$ precipitate to form; then this was removed by centrifugation. A control flask containing Krebs–Henseleit saline plus additions but no tissue was treated in the same manner.

The neutralized samples were used for metabolite assays. The tissue extract was assayed for glucose 6-phosphate and ATP (Lamprecht & Trautschold, 1963), ADP (Adam, 1963) and citrate (Möllering & Gruber, 1966). The medium was assayed for glucose (Slein, 1963), pyruvate and lactate (Hohorst *et al.*, 1959), and acetoacetate and 3-hydroxybutyrate (Williamson *et al.*, 1962) by standard enzymic techniques.

Glucose removal was calculated from the difference between the glucose present in the control and that remaining in experimental flasks after 1h incubation period, and expressed in μmol/h per g wet wt. Metabolite concentrations are expressed in μmol/g wet wt.

In separate experiments O_2 uptake was found to be linear for at least 90min (M. P. Thompson & D. H. Williamson, unpublished work).

Radioactivity experiments. Slices (200±10mg wet wt.) were incubated for 1h at 37°C in 50ml Erlenmeyer flasks fitted with 1ml centre wells, in Krebs–Henseleit (1932) bicarbonate saline (5ml) containing the appropriate additions: [6-^{14}C]glucose (5mM) or [3-^{14}C]pyruvate (2mM), acetoacetate (2.5mM) and adrenaline (10μg/ml), gassed with O_2+CO_2 (95:5) and sealed with Suba-seals (A. Gallenkamp and Co., London EC2P 2ER, U.K.). The rates of $^{14}CO_2$ production were almost linear for 1h in these experiments. After 1h 30% (w/v) $HClO_4$ (0.5ml) was injected through the seal into the medium and Hyamine (0.5ml) was injected into the centre well. Shaking was continued for a further 2h at room temperature to absorb the CO_2 into the Hyamine. The Hyamine was then quantitatively removed and samples were counted for radioactivity in a Beckman liquid-scintillation counter in a scintillation fluid of the following composition: 8g of butyl-PBD, 60g of naphthalene in 2-methoxyethanol (400ml) and toluene (600ml).

The amount of glucose (μmol) oxidized to CO_2 was estimated by dividing the total radioactivity in CO_2 (c.p.m.) by the specific radioactivity of the glucose in the initial medium. The specific radioactivity (c.p.m./μmol) was determined by measuring the initial total c.p.m. and the initial total glucose in the medium.

Experiments in vivo. The rats were anaesthetized with Nembutal (60mg/kg body wt.) dissolved in 0.9% NaCl. A ventral midline incision was made in the neck. The submaxillary glands were dissected free of surrounding tissue and any adhering fatty material was removed. The right-hand-side gland

Table 1. *Effects of acetoacetate and adrenaline on glucose metabolism by submaxillary-gland slices*

Submaxillary-gland slices (200mg wet wt.) were incubated for 1h at 37°C in Krebs–Henseleit (1932) bicarbonate saline (5ml) gassed with O_2+CO_2 (95:5), and with appropriate additions: glucose (5mM), acetoacetate (2.5mM) and adrenaline (10μg/ml). Metabolite determinations were carried out as described under 'Methods'. The results are expressed in μmol/h per g wet wt. or μmol/g wet wt.±S.D., with the number of experiments indicated in parentheses. Values that are statistically different from the glucose control by the Student's t test are indicated by *: $P<0.005$.

Additions	Glucose removal	Lactate formed	Pyruvate formed	[Lactate]/[pyruvate]	Lactate+pyruvate formed	Acetoacetate removal	3-Hydroxybutyrate formed
Glucose	25.4±4.3 (23)	10.4±2.0 (8)	1.19±0.27 (8)	8.3±0.9 (6)	11.6±2.1 (8)	—	—
Glucose+adrenaline	31.3±7.7 (20)*	15.4±2.6 (6)*	0.62±0.25 (6)*	23.8±8.1 (5)*	16.0±2.7 (6)*	—	—
Glucose+acetoacetate	19.8±4.6 (15)*	12.7±2.4 (7)	1.28±0.21 (7)	10.4±2.1 (7)	13.9±2.3 (7)*	20.4±3.3 (21)	2.85±0.75 (18)
Glucose+acetoacetate +adrenaline	33.0±4.9 (14)*	19.0±2.5 (6)*	1.73±0.53 (5)	9.8±0.9 (4)	20.6±2.8 (6)*	20.0±2.8 (18)	4.07±0.38 (6)*

was chosen as the control for all experiments. A fine suture was placed through the apex of the gland so that the gland could be held clear of other tissue yet still maintaining the blood supply as the artery enters at the base of the gland. At 10min after dissection the control gland was clamped between aluminium tongs that had been cooled in liquid N_2 (Wollenberger *et al.*, 1960). The artery supplying the gland was tied to prevent blood loss before the frozen gland was cut free. Then isoproterenol (0.2ml, 2mg) or 0.9% NaCl (0.2ml) was injected into a femoral vein. At 10min after this treatment the collateral experimental gland was freeze-clamped. The frozen tissue (about 0.2g) was powdered in liquid N_2 with a pestle and mortar. The tissue was weighed and the metabolites were extracted into 3% $HClO_4$ (5ml) by homogenizing with a motor-driven Teflon homogenizer. This extract was centrifuged for 1h at 30000g at 4°C.

The supernatant was neutralized as described above for the incubation medium, and this solution was used for metabolite measurements.

Results

Effects of acetoacetate on glucose metabolism

When acetoacetate (2.5mM) was present in the incubation medium the rate of glucose removal by submaxillary-gland slices was decreased by 22% (Table 1). DL-3-Hydroxybutyrate (5mM) decreased glucose removal by a similar amount (results not shown). Measurement of the formation of $^{14}CO_2$ from [6-^{14}C]glucose showed that acetoacetate not

Table 2. *Effects of acetoacetate and adrenaline on $^{14}CO_2$ production from [6-^{14}C]glucose by submaxillary-gland slices*

Submaxillary-gland slices (200mg wet wt.) were incubated for 1h at 37°C in Krebs–Henseleit (1932) bicarbonate saline (5ml) gassed with O_2+CO_2 (95:5), and with appropriate additions: [6-^{14}C]glucose (5mM; 6μCi/mmol), acetoacetate (2.5mM), adrenaline (10μg/ml). The $^{14}CO_2$ was collected and counted for radioactivity as described under 'Methods'. The results are expressed as μmol of [6-^{14}C]glucose converted into $^{14}CO_2$/h per g wet wt.±S.D., with the number of experiments indicated in parentheses. Values that are statistically different from the glucose only values by the Student's t test are indicated by *: $P<0.001$.

Additions	[6-^{14}C]Glucose converted into $^{14}CO_2$
Glucose	6.2±0.65 (7)
Glucose+adrenaline	10.9±1.45 (6)*
Glucose+acetoacetate	3.5±0.21 (4)*
Glucose+acetoacetate +adrenaline	8.6±1.15 (3)*

Table 3. *Effects of acetoacetate and adrenaline on metabolite concentrations in submaxillary-gland slices*

Submaxillary-gland slices (200mg wet wt.) were incubated for 1h at 37°C in the Krebs–Henseleit bicarbonate saline (5ml) gassed with O_2+CO_2 (95:5), and with appropriate additions: glucose (5mM), acetoacetate (2.5mM) and adrenaline (10μg/ml). At the end of the incubation the slices were removed from the medium and assayed for metabolites as described under 'Methods'. All concentrations are expressed in μmol/g wet wt.+S.D., with the number of experiments indicated in parentheses. Values that are statistically different from slices incubated in glucose alone by the Student's t test are indicated by *: $P<0.01$.

Additions	[Glucose 6-phosphate]	[Citrate]	[ATP]	[ADP]
Glucose	0.093±0.036 (16)	0.132±0.072 (12)	0.651±0.138 (20)	0.164±0.055 (7)
Glucose+adrenaline	0.027±0.022 (18)*	0.154±0.065 (12)	0.330±0.095 (23)*	0.213±0.032 (8)
Glucose+acetoacetate	0.122±0.024 (17)*	0.299±0.096 (13)*	0.691±0.111 (24)	0.159±0.022 (7)
Glucose+acetoacetate +adrenaline	0.058±0.026 (13)*	0.285±0.068 (13)*	0.317±0.114 (23)*	0.215±0.044 (7)

Table 4. *Effects of fluoroacetate on glucose metabolism by submaxillary-gland slices*

Submaxillary-gland slices (200mg wet wt.) were incubated for 1h at 37°C in the Krebs–Henseleit bicarbonate saline (5ml) gassed with O_2+CO_2 (95:5), and with appropriate additions: glucose (5mM), fluoroacetate (0.2mM) and adrenaline (10μg/ml). Determinations were carried out as described under 'Methods'. The results are expressed in μmol/h per g wet wt. or μmol/g wet wt.±S.D., with the number of experiments indicated in parentheses. Values that are statistically different from the glucose control by the Student's t test are indicated by *: $P<0.005$.

Additions	Glucose removal	[Citrate]	[Glucose 6-phosphate]
Glucose	27.0±3.5 (8)	0.185±0.06 (4)	0.096±0.03 (6)
Glucose+adrenaline	35.1±4.4 (6)*	0.265±0.09 (4)	0.039±0.02 (3)*
Glucose+fluoroacetate	20.4±2.8 (8)*	2.86 ±2.80 (6)*	0.202±0.058 (6)*
Glucose+fluoroacetate+adrenaline	26.5±5.4 (8)	3.15 ±1.09 (6)*	0.105±0.046 (6)

only inhibited glucose removal but also inhibited entry of glucose carbon into the tricarboxylic acid cycle, as shown by a 45% decrease in radioactive CO_2 (Table 2). A significant increase ($P<0.005$) in the lactate+pyruvate pool is consistent with this finding (Table 1).

The concentrations of some metabolites in the slices were measured (Table 3) to help determine the site(s) of inhibition of glucose metabolism. The adenine nucleotide concentration within the slices did not change significantly when acetoacetate was present in the incubation medium. However, a significant increase ($P<0.01$) in tissue [citrate] and [glucose 6-phosphate] was observed. These findings suggested that acetoacetate was acting on glycolysis via the elevation of [citrate], which can inhibit phosphofructokinase and thus cause the rise in [glucose 6-phosphate], which in turn might decrease glucose phosphorylation.

Additional evidence for this mechanism was obtained by incubating slices with fluoroacetate to elevate [citrate]. Tissue [citrate] was increased 15-fold by fluoroacetate (Table 4), owing to competitive inhibition of aconitase by fluorocitrate (Morrison & Peters, 1954). This rise in [citrate] was accompanied by an increase in [glucose 6-phosphate] and a 25% fall in glucose removal (Table 4), which supports the suggestion that [citrate] can modulate phosphofructokinase activity in submaxillary-gland tissue.

The increase in lactate+pyruvate pool and the inhibition of oxidation of [6-^{14}C]glucose to $^{14}CO_2$ in the presence of acetoacetate suggested a second site of inhibition at the stage of pyruvate oxidation. To look specifically at this step experiments were carried out with pyruvate as substrate (Table 5). Acetoacetate inhibited pyruvate removal by 22%, thus implicating pyruvate dehydrogenase as another site of action of acetoacetate. That the elevated [citrate] is not responsible for the acetoacetate inhibition is indicated by the finding that fluoroacetate had no effect on pyruvate uptake (results not shown). It has been reported that elevated [acetyl-CoA] can inhibit pyruvate dehydrogenase from pig heart (Garland & Randle, 1964) and this may also be a mechanism in submaxillary-gland tissue.

Effects of adrenaline on glucose metabolism

Adrenaline is a stimulator of secretion in this tissue (Byrt & Glanvill, 1967; Pritchard, 1972) and therefore it was decided to see whether it could modify the interactions between glucose and ketone bodies. When adrenaline was added to the incubation medium at concentrations (10μg/ml) reported to

stimulate secretion of amylase *in vitro* (Ueha *et al.*, 1971), a 25% increase of glucose removal compared with the control value was observed in the absence or presence of acetoacetate (Table 1). Thus it appears that adrenaline not only stimulates glucose removal but can also overcome the inhibition of glucose utilization caused by acetoacetate. No alteration of the amount of acetoacetate removed was observed with adrenaline (Table 1). About 15% of the acetoacetate removed from the medium is converted into 3-hydroxybutyrate and this increased to about 20% in the presence of adrenaline.

Adrenaline caused a proportionate increase in the lactate+pyruvate pool formed when glucose was the sole substrate and did not prevent the increased accumulation of these metabolites when acetoacetate was present (Table 1). On the other hand, adrenaline did overcome to a large extent the inhibition of pyruvate removal and its oxidation to CO_2 when acetoacetate was present (Table 5).

It is probable that adrenaline also stimulated glycogenolysis in these experiments, but as the [glycogen] of freeze-clamped gland is about 4 μmol of glucose equivalents/g it is not likely to make any major contribution to glycolysis in this situation.

Measurement of tissue metabolite concentrations showed a decrease in [glucose 6-phosphate] with adrenaline, compared with the increase observed in the presence of acetoacetate (Table 3), which is indicative of relief of inhibition at the phosphofructokinase step. Inhibition of glucose removal by fluoroacetate was also overcome by adrenaline (Table 4). However, adrenaline had no effect on the elevation of [citrate] brought about by either fluoroacetate or acetoacetate (Tables 3 and 4), which is in accord with the unchanged rate of acetoacetate utilization. Measurement of adenine nucleotide concentrations showed that adrenaline caused a 50% decrease in tissue [ATP] in either the presence or the absence of acetoacetate (Table 3), suggesting that the mechanism for stimulating glucose removal and relieving inhibition by acetoacetate was associated with this effect. A rapid fall in tissue [ATP] on addition of adrenaline to preparations of rat parotid glands *in vitro* has also been observed by Batzri & Selinger (1973).

Effects of adrenergic stimulation on ATP concentration in vivo

To test whether the marked decrease in [ATP] occurred only in experiments *in vitro*, submaxillary glands were subjected to adrenergic stimulation

Table 5. *Effects of acetoacetate and adrenaline on pyruvate metabolism by submaxillary-gland slices*

Submaxillary-gland slices (200mg wet wt.) were incubated for 1h at 37°C in the Krebs–Henseleit bicarbonate saline (5ml) gassed with O_2+CO_2 (95:5) and with appropriate additions: pyruvate (2mM) or [3-^{14}C]pyruvate (2mM; 30μCi/mmol), acetoacetate (2.5mM) and adrenaline (10μg/ml). The results are expressed in μmol/h per g wet wt. or μmol/g wet wt. ± S.D., with the number of experiments indicated in parentheses. Values that are statistically different from the pyruvate control by the Student's *t* test are indicated by *: $P<0.001$.

Additions	Pyruvate removal	[3-^{14}C]Pyruvate converted into $^{14}CO_2$	Lactate formation	Tissue [ATP]
Pyruvate	38.4±3.0 (12)	6.29±0.68 (4)	7.70±0.57 (12)	0.833±0.188 (18)
Pyruvate+adrenaline	39.6±3.2 (8)	7.76±0.56 (4)	7.14+0.46 (8)	0.543±0.46(4)*
Pyruvate+acetoacetate	29.9±2.7 (8)*	4.44±0.01 (2)	11.4±0.89 (8)*	0.956±0.111 (11)
Pyruvate+acetoacetate +adrenaline	35.8±3.8 (6)	7.03±0.01 (2)	9.47±1.82 (6)	0.531±0.068 (6)*

Table 6. *Effect of isoproterenol injection on ATP concentrations in rat submaxillary glands in vivo*

A total of eight rats was used. The rats were anaesthetized and the submaxillary glands dissected free from surrounding tissue as described under 'Methods'. The right-hand-side gland (RHS) was chosen as the control for all experiments. This was freeze-clamped 10min after dissection. Control tissue samples were obtained by clamping the remaining collateral gland (LHS) 10min after intravenous injection of 0.9% NaCl (0.2ml). Experimental tissue samples were obtained from separate rats, by clamping the collateral gland after intravenous injection of isoproterenol (2mg, 0.2ml). Values are means ± S.D., for the numbers of experiments given in parentheses and those that are statistically different from the control gland (RHS) by the Student's *t* test are indicated by *: $P<0.001$.

	Experimental treatment of rats	Number of glands	[ATP] (μmol/g wet wt.)	[Lactate]/[Pyruvate]
Control gland (RHS)	None	8	1.30±0.33	12.0±3.5 (8)
Experimental control gland (LHS)	Saline injection	3	1.36±0.24	11.7±1.7 (2)
Experimental gland (LHS)	Isoproterenol injection	5	0.91±0.40*	10.1±2.4 (3)

Table 7. *Effects of dibutyryl cyclic AMP on glucose metabolism and ATP content in rat submaxillary-gland slices*

Submaxillary-gland slices (200mg wet wt.) were incubated for 1h at 37°C in the Krebs–Henseleit bicarbonate saline (5ml) gassed with O_2+CO_2 (95:5), and with appropriate additions: glucose (5mM), acetoacetate (2.5mM) and dibutyryl cyclic AMP (Bu_2cAMP; 1mM). Metabolite determinations were carried out as described under 'Methods'. Results are expressed as means±S.D., with the number of experiments indicated in parentheses. Values that are statistically different from the glucose control by the Student's t test are indicated by *: $P<0.05$.

Additions	Glucose uptake (μmol/h per g wet wt.)	Pyruvate formed (μmol/h per g wet wt.)	Lactate formed (μmol/h per g wet wt.)	Tissue [ATP] (μmol/g wet wt.)
Glucose	28.8±7.8 (11)	1.11±0.20 (8)	7.39±0.92 (8)	0.757 (3)
Glucose+Bu_2cAMP	30.8±7.5 (12)	1.06±0.17 (8)	8.27±0.85 (8)	0.721 (4)
Glucose+acetoacetate	23.1±6.0 (10)*	1.26±0.23 (8)	9.59±1.74 (8)	0.721 (4)
Glucose+acetoacetate +Bu_2cAMP	24.3±3.0 (10)*	1.43±0.19 (8)	11.4 ±3.04 (8)	0.770 (4)

in vivo and the [ATP] was measured after freeze-clamping the glands. The values found for normal glands (Table 6) were about twice those obtained in the slice experiments and were in good agreement with the values measured *in vivo* by Dreisbach & Gerlach (1967). Higher [ATP] values for the experiments *in vitro* have recently been obtained by dropping the slices into liquid N_2 at the end of the incubation period (M. P. Thompson & D. H. Williamson, unpublished work). Isoproterenol administration *in vivo* caused a 30% decrease in [ATP] (Table 6). This change occurred without any significant alteration in the [lactate]/[pyruvate] ratio, which rules out hypoxia as the reason for the fall in [ATP]. Isoproterenol was used for these experiments because it is less toxic than adrenaline (Byrt, 1966) and it has similar effects *in vitro* to adrenaline.

Effects of dibutyryl cyclic AMP on glucose metabolism

It has been postulated that adrenaline stimulates secretion via an increase in cyclic AMP in the parotid gland (Bdolah & Schramm, 1965). If the metabolic action of adrenaline in the submaxillary gland is also exerted through this mechanism, it might be expected that its effects on glucose metabolism could be reproduced by cyclic AMP. In the present experimental system dibutyryl cyclic AMP had no effect on glucose removal nor was it able to overcome the inhibitory effect of acetoacetate on this process (Table 7). Tissue [ATP] was also unaffected by dibutyryl cyclic AMP (Table 7). These findings raise the question whether the metabolic effects of adrenaline are mediated by mechanisms other than by changes in [cyclic AMP].

Effects of acetoacetate and adrenaline on glucose metabolism in a Ca^{2+}-depleted medium

From the reports of several workers (Rasmussen & Tenenhouse, 1968; Douglas & Poisner, 1963) it appears that secretion from salivary-gland tissue requires the presence of Ca^{2+}. To test whether the metabolic effects of adrenaline were also dependent on the availability of Ca^{2+} the experiments reported in Table 1 were repeated in a medium from which Ca^{2+} was omitted. The control rate of glucose removal was lower than in the complete medium, yet acetoacetate still inhibited removal by 15%, and adrenaline stimulated removal by 27% (Table 8). However, when glucose and acetoacetate were both present in the incubation medium, adrenaline no longer stimulated glucose removal beyond the control value, compared with 30% stimulation when Ca^{2+} was present.

Measurements of metabolite concentrations (Table 8) within the slices showed that when adrenaline was present [glucose 6-phosphate] did not fall below the control value, compared with a 70% fall in the Ca^{2+}-containing medium. The citrate concentrations were similar to those found in experiments with the complete medium. The ATP concentrations were slightly lower than those in slices incubated in the Ca^{2+}-containing medium, but the presence of adrenaline did not lower [ATP] significantly below the control value.

The utilization of pyruvate was also studied in the medium from which Ca^{2+} had been omitted (Table 9). Acetoacetate inhibited pyruvate removal, but in the absence of Ca^{2+} ions adrenaline was no longer able to overcome this inhibition. This was confirmed by a single experiment using radioactively labelled [3-^{14}C]pyruvate, which showed that the decrease in $^{14}CO_2$ production caused by acetoacetate was not reversed when adrenaline was present.

The finding of Sandhu *et al.* (1964) that noradrenaline only increased $^{14}CO_2$ production from [1-^{14}C]glucose when submaxillary-gland slices were incubated in the presence of Ca^{2+} also emphasizes the importance of Ca^{2+} for the stimulation of glucose oxidation by adrenergic drugs in this tissue.

Restoration of Ca^{2+} to the medium re-established the metabolic patterns previously observed.

Table 8. *Effects of acetoacetate and adrenaline on glucose metabolism and some metabolite concentrations in submaxillary-gland slices incubated in a Ca^{2+}-depleted medium*

Submaxillary-gland slices (200mg wet wt.) were incubated for 1h at 37°C in the Krebs–Henseleit bicarbonate saline (5ml) from which Ca^{2+} had been omitted ($CaCl_2$ replaced by an equivalent amount of NaCl), gassed with O_2+CO_2 (95:5), and with appropriate additions: glucose (5mM), acetoacetate (2.5mM), adrenaline (10μg/ml) and Ca^{2+} (2.9mM). The results are expressed in μmol/h per g wet wt. or μmol/g wet wt.±S.D., with the number of experiments indicated in parentheses. Values that are statistically different from the glucose control by the Student's *t* test are indicated by *: $P<0.005$.

Additions	Glucose uptake	Lactate+pyruvate formed	[Glucose 6-phosphate]	[Citrate]	[ATP]
Glucose	22.5±2.75 (11)	11.5±1.90 (8)	0.071±0.030 (11)	0.131±0.038 (10)	0.545±0.147 (9)
Glucose+adrenaline	28.7±2.84 (9)*	15.7±2.98 (7)*	0.065±0.038 (11)	0.157±0.057 (13)	0.473±0.172 (11)
Glucose+acetoacetate	18.9±1.13 (12)*	13.2±1.68 (7)	0.126±0.036 (12)*	0.269±0.062 (12)*	0.663±0.095 (12)
Glucose+acetoacetate+adrenaline	22.2±2.92 (10)	18.0±2.72 (8)*	0.080±0.024 (12)	0.298±0.092 (14)*	0.552±0.117 (13)
Glucose+acetoacetate+adrenaline +$CaCl_2$	29.1 (2)	17.9 (4)	0.016 (6)	0.196 (6)	0.330 (12)

Action of other hormones and effectors

A number of other hormones and possible effectors were tested for their action in the submaxillary-gland-slice system. Neither glucagon nor insulin had any effect. Isoproterenol and noradrenaline, which can also stimulate secretion behaved in a way similar to adrenaline. Theophylline had no effect.

Discussion

Glucose and acetoacetate interactions

Glucose removal from the incubation medium and entry of glucose carbon into the tricarboxylic acid cycle are inhibited by physiological concentrations of acetoacetate (Tables 1 and 2). It has also been reported that pretreatment of rat submaxillary gland slices with palmitic acid inhibits $^{14}CO_2$ production from [U-^{14}C]glucose by approximately 20% (Pritchard, 1972). These findings extend to another tissue the observations that are now generally accepted, namely that fatty acids and ketone bodies inhibit glucose utilization by extrahepatic tissues as has been demonstrated for example in rat heart and diaphragm (Randle *et al.*, 1964). However, it should be pointed out that the inhibitory effect of acetoacetate on glucose uptake by submaxillary gland (mean, 22% decrease) is considerably less than that observed with perfused heart at the same [acetoacetate] (Randle *et al.*, 1964; about 50–60% decrease). Higher [acetoacetate] (up to 10mM) did not increase the degree of inhibition in submaxillary gland. The inhibition brought about by fluoroacetate (Table 4) is also much less than that observed by Bowman (1964) in the perfused rat heart.

In the rat heart and diaphragm the mechanism for the inhibition of glucose utilization appears to be associated with effects at three intracellular sites: hexokinase, phosphofructokinase and pyruvate dehydrogenase (Randle *et al.*, 1964). Measurements of changes in metabolite concentrations within the submaxillary gland slices and in the medium suggests that these same sites are involved in this tissue.

Substrate–hormone interactions

There have been no reports that similar substrate interactions to those described above for submaxillary-gland tissue can be modified by the direct action of hormones. So, from the present study one of the most noteworthy findings is that adrenaline can directly modulate the metabolic interactions of key substrates; adrenaline increased glucose utilization and abolished the inhibitory effect of acetoacetate on glucose removal.

The only report of stimulation of glucose utilization in submaxillary glands by adrenaline showed 60% increase of $^{14}CO_2$ formation from

Table 9. *Effects of acetoacetate and adrenaline on pyruvate metabolism by submaxillary-gland slices incubated in a Ca^{2+}-depleted medium*

Submaxillary-gland slices (200mg wet wt.) were incubated for 1h at 37°C in the Krebs–Henseleit bicarbonate saline (5ml) from which Ca^{2+} had been omitted, gassed with O_2+CO_2 (95:5), and with appropriate additions: pyruvate (2mM) or [3-^{14}C]pyruvate (2mM, 30μCi/mmol), acetoacetate (2.5mM) and adrenaline (10μg/ml). The results were obtained as described under 'Methods', and expressed in μmol/h per g wet wt. as means±S.D. of the number of experiments indicated in parentheses, except where the results are the means of one experiment carried out in duplicate. Values that are statistically different from control by the Student's *t* test are indicated by *: $P<0.01$.

Additions	Pyruvate uptake	[3-^{14}C]Pyruvate converted into $^{14}CO_2$	Lactate formation
Pyruvate	34.0±3.1 (6)	7.78	9.43±1.17 (6)
Pyruvate+adrenaline	31.8±4.8 (2)	8.68	9.28±0.15 (2)
Pyruvate+acetoacetate	28.3±2.6 (4)*	5.84	9.96±1.54 (4)
Pyruvate+acetoacetate+adrenaline	25.7±4.5 (2)*	5.87	10.94±0.54 (2)

[6-^{14}C]glucose (Ueha *et al.*, 1971), which is in good agreement with the results reported here. However, there have been no reports of measurements of the absolute glucose removal, except by Feinstein & Schramm (1970) who showed a very low rate of glucose uptake by parotid slices (about 20% of the values reported here).

Adrenaline has been reported to stimulate glucose uptake in several other extrahepatic tissues; heart muscle (Williamson, 1966), thyroid gland (Pastan *et al.*, 1962), and adipose tissue (Leboeuf *et al.*, 1959; Vaughan, 1961). In the case of diaphragm there are conflicting reports about the effects of adrenaline (Walaas & Walaas, 1950; Herman & Ramey, 1960). Adrenaline did not stimulate glucose oxidation in mouse pancreatic islets (Ashcroft *et al.*, 1970). The mechanism whereby adrenaline causes the stimulatory effect is still to be elucidated in these tissues, but all suggestions stress the relationship to the specific tissue function stimulated by adrenaline.

One site of action of adrenaline on glucose metabolism in the submaxillary gland appears to be at or below the phosphofructokinase step in glycolysis. This is indicated by the decrease in tissue [glucose 6-phosphate] which accompanies the increase in glucose utilization (Tables 1 and 3).

The negligible activity of the pentose phosphate pathway in submaxillary gland (Goldman *et al.*, 1964) and the fact that adrenaline does not alter the proportion of $^{14}CO_2$ derived from [6-^{14}C]glucose and [1-^{14}C]glucose (Ueha *et al.*, 1971) rules out the fall in [glucose 6-phosphate] being due to stimulation of the pentose phosphate pathway by adrenaline.

A possible explanation for the increased glycolytic flux in the presence of adrenaline is the decreased [ATP] (Table 3) which might be expected to activate phosphofructokinase. However, the finding of a similar increase in glucose utilization with a minimal fall in [ATP] in the Ca^{2+}-depleted medium does not support this postulate. Somewhat more certain is that the fall in [ATP] brought about by adrenaline is responsible for the abolition of the inhibition of glucose utilization by acetoacetate, because this did not occur in the Ca^{2+}-depleted medium where [ATP] did not decrease significantly. Thus it would appear that the primary effect of acetoacetate is to increase [citrate] which inhibits phosphofructokinase and decreases glycolytic flux. Adrenaline in the presence of Ca^{2+} ions lowers [ATP] which in turn relieves, most likely, the inhibition at the phosphofructokinase step.

A second site where glucose, acetoacetate and adrenaline interact is the pyruvate dehydrogenase step. Pyruvate dehydrogenase is an interconvertible enzyme regulated by a phosphorylation–dephosphorylation cycle, being phosphorylated by a kinase to the inactive form and dephosphorylated by a phosphatase to the active form (Linn *et al.*, 1969*a*,*b*). It is possible that adrenaline abolishes the acetoacetate inhibition of pyruvate dehydrogenase by decreasing the activity of the kinase via the fall in [ATP] and thus causing an increase in the proportion of active enzyme. When Ca^{2+} is absent from the medium then activation by this mechanism might not occur because the activity of the phosphatase could be low (Denton *et al.*, 1972; Severson *et al.*, 1974) and the decrease in [ATP] minimal.

Effect of adrenaline on ATP concentration

The interaction of adrenaline with glucose metabolism at both the phosphofructokinase step and the pyruvate dehydrogenase step appears to depend at least in part on the fall in [ATP]. The mechanism that brings about this fall in [ATP] is still to be elucidated, but it may be related to the secretory function of the tissues which is stimulated by adrenaline. [ATP] did not fall when Ca^{2+} was absent from the medium. Several mechanisms of secretion have been postulated which involve a (Na^++K^+)-activated ATPase (adenosine triphos-

phatase) and/or a Ca^{2+}-dependent ATPase (Schwartz & Matsui, 1967; Selinger *et al.*, 1970). The result of stimulation of the ATPase systems by adrenaline in the presence of Ca^{2+} could be consumption of ATP. The extrusion of zymogen granules during the secretory process (Amsterdam *et al.*, 1969) may also be an energy-requiring process and thus diminish [ATP].

An alternative mechanism for the decrease in [ATP] comes from studies on adipocytes where it is postulated that adrenaline increases the intracellular free fatty acid concentration by stimulating lipolysis with consequent uncoupling of oxidative phosphorylation and decrease in [ATP] (Bihler & Jeanrenaud, 1970; Angel *et al.*, 1971). It would seem unlikely that this mechanism is operative in salivary glands because of the rapidity (within 1 min) of the [ATP] fall (Batzri & Selinger, 1973) and the finding that it does not occur with added dibutyryl cyclic AMP or theophylline (stimulators of lipolysis). The fact that submaxillary gland [ATP] also decreases when the tissue is subjected to adrenergic stimulation *in vivo* suggests that the observation *in vitro* may be of physiological interest.

Although secretion was not measured in these experiments it is well-established that adrenaline enhances this process. A question still to be answered is the interrelationship between the stimulation of the metabolic and secretory events brought about by adrenaline.

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