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A comparison of the effects on blood glucose and ketone-body levels, and of the toxicities, of pent-4-enoic acid and four simple fatty acids

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Some effects of the hypoglycaemic compound pent-4-enoic acid in rats and mice, are described. Pent-2-enoic acid, pentanoic acid, cyclopropanecarboxylic acid and cyclobutanecarboxylic acid, which were shown to be non-hypoglycaemic, were used as controls. Pent-4-enoic acid and cyclopropanecarboxylic acid caused ketosis in rats, with a lowering of the blood β-hydroxybutyrate/acetocetate (βHB/AcAc) ratio; some ketosis was caused by the other fatty acids but the βHB/AcAc ratio was not changed. Pent-4-enoic acid caused an increase in free fatty acid concentration in rat plasma. The acute toxicities of these compounds in mice were determined. The mechanism of the hypoglycaemic action of pent-4-enoic acid is discussed in relation to that of hypoglycin.

Pent-4-enoic acid has been reported briefly to be hypoglycaemic (Anderson, Johnson & others, 1958; McKerns, Bird & others, 1960; Senior & Sherratt, 1966). It is the simplest member of a series of hypoglycaemic fatty acids related to hypoglycin (L-α-amino-β-methylenecyclopropanepropionic acid) (Anderson & others, 1958), the toxic principle of the unripe ackee fruit, *Blighia sapida* (Hassall, Reyle & Feng, 1954). The structural requirement for hypoglycaemic activity is a vinyl group separated from a carboxyl group by two carbon atoms.

In this paper some effects of pent-4-enoic acid in animals are compared with those of four non-hypoglycaemic fatty acids which were used as controls. Pent-2-enoic acid and pentanoic acid were used because of their structural relationship to pent-4-enoic acid; and cyclopropanecarboxylic and cyclobutanecarboxylic acid were used because of their relationship to the hypoglycaemic compounds, methylenecyclopropaneacetic acid and 3-methylenecyclobutanecarboxylic acid (Anderson & others, 1958). These four control fatty acids were also used in a biochemical investigation of the mechanism of action of pent-4-enoic acid in order to establish which *in vitro* effects are correlated with its hypoglycaemic activity (Senior & Sherratt, 1968a, b; Senior, Robson & Sherratt, 1968). The effects of the fatty acids on blood ketone bodies were studied since both hypoglycin and cyclopropanecarboxylic acid were known to cause a strong ketosis in rats (Williamson & Wilson, 1965).

EXPERIMENTAL

Materials

Butyric acid, pentanoic acid, hexanoic acid, crotonic acid and pent-4-enoic acid (Fluka A. G. Buchs, Switzerland), acrylic acid, pent-2-enoic acid, hex-3-enoic acid,

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hept-2-enoic acid, sorbic acid, DL-carnitine, riboflavin and riboflavin phosphate (Koch-Light Laboratories Ltd., Colnbrook, Bucks.), cyclopropanecarboxylic acid and cyclobutanecarboxylic acid (Aldrich Chemical Co., Milwaukee, Wisconsin, U.S.A.). Glucose oxidase kits for glucose estimation, NAD^+, NADH and crystalline D-β-hydroxybutyrate dehydrogenase (EC 1.1.1.30) 140 units/mg, were obtained from C. F. Boehringer und Soehne, G.m.b.H., Mannheim, Germany. Fatty acids were injected as aqueous solutions of their sodium salts, pH 7·4; the doses quoted refer to the free acids.

Animals

All animals used were albino males, the rats were Wistar strain.

Methods

Effects of pent-4-enoic acid and related fatty acids on blood glucose levels. Rats (150–200 g), mice (22–26 g) and rabbits (1·5–3·0 kg) were starved for 24 h to deplete their glycogen reserves and food was withheld during the experiments. Fatty acids or 0·153 M NaCl were given to rats intraperitoneally and to mice and rabbits subcutaneously. Blood was taken from the tails of rats or the ear veins of rabbits at intervals and collected in cold heparinized tubes. Mice were killed at intervals after injection and blood was collected from the heart. Blood was deproteinized with 0·6 N $HClO_4$ and glucose was determined using glucose oxidase (EC 1.1.3.4) with test-kits supplied by Boehringer. Samples taken at the nadir of hypoglycaemia gave very low optical density readings, so the reaction mixture was fortified with additional potassium phosphate to give a final concentration of 0·4 M, pH 7·0. Larger samples of the acid supernatant could then be assayed.

RESULTS

Effects of pent-4-enoic acid and related fatty acids on blood glucose levels

Rats. Only pent-4-enoic acid caused marked hypoglycaemia (Table 1), the time course of which is illustrated in Fig. 1. There was some individual variation in the

Table 1. *Blood glucose levels in rats after administration of pent-4-enoic acid and related fatty acids.* Blood glucose levels were determined in each animal at 1 h intervals for at least 7 h as described in the Methods section. Zero time blood glucose concentration ± s.d. were (mM) 3·33 ± 0·75 (41).

Fatty acid	Dose (mg/kg)	No. of animals	Range of blood glucose levels (mM) observed during experiments	Mean values ± s.d. or range of greatest percentage changes in, blood glucose levels	P where applicable
Pent-4-enoic acid	100	6	1·45–3·65	—23·2 ± 8·0	>0·001
	150*	6	1·74–4·00	—35·7 ± 8·6	>0·001
	200*	6	1·28–4·67	—41·9 ± 21·7	>0·01 <0·001
	250*	3	0·02–3·05	—79 ± 34·5	>0·01
Pent-2-enoic acid	50–250	3	2·50–3·50	0– —20	—
n-Pentanoic acid	50–250	3	3·22–3·88	0–+20	—
Cyclopropanecarboxylic acid	50–250	6	3·77–4·02	0– —18	Not significant
Cyclobutanecarboxylic acid	50–280	3	3·44–4·72	+23–+61	—
Control (given 0·153 M NaCl)	—	5	2·77–4·56	0–+24	—

* One death.

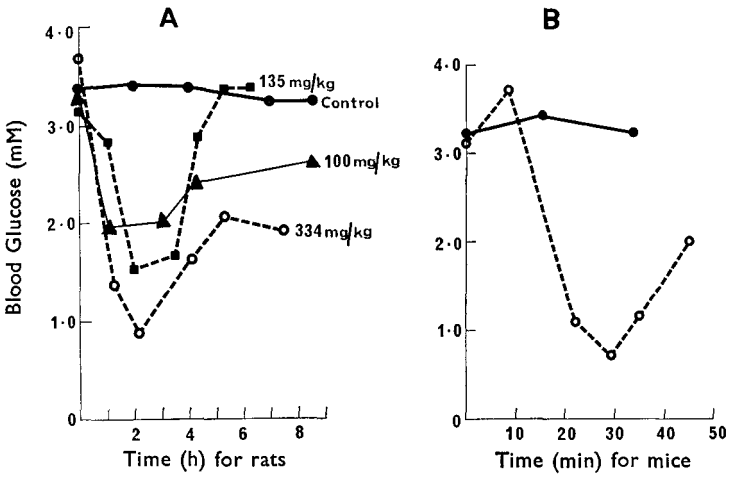

FIG. 1A. Time course of hypoglycaemia in individual rats caused by intraperitoneal administration of pent-4-enoic acid; control (●), pent-4-enoic acid 100 mg/kg (▲), pent-4-enoic acid 135 mg/kg (■), pent-4-enoic acid 334 mg/kg (○).

B. Hypolgycaemia in mice caused by subcutaneous administration of pent-4-enoic acid (1·0 g/kg); control (●), pent-4-enoic acid (○). Each point represents the blood glucose level in one animal.

hypoglycaemic effect, and pent-4-enoic acid ($M = 100$) was approximately half to a third as effective on a weight basis in producing maximum hypoglycaemia as hypoglycin ($M = 141$) (Feng & Patrick, 1958). Pent-2-enoic acid in large doses (250 mg/kg) caused a slight fall (20%) in blood glucose. All fatty acids caused depression and irregular respiration but pent-4-enoic acid was markedly most depressant. Similar depressant effects are caused by injection of saturated short-chain fatty acids (Samson & Dahl, 1955; Samson, Dahl & Dahl, 1956). Rats that recovered from hypoglycaemia appeared normal after 24 h and also after six months.

Mice. Pent-4-enoic acid caused pronounced hypoglycaemia in mice (Fig. 2). All treated animals became severely depressed after 15 min. Breathing was difficult and was deep and irregular, with epistaxis, exophthalmos and loss of righting reflex.

Rabbits. There were no consistent or significant effects on blood glucose levels for up to 28 h after injections of pent-4-enoic acid (30–425 mg/kg) in 14 rabbits. The lethal dose was variable (200–425 mg/kg) but the blood glucose did not fall even at the time of death, and in one animal it rose sharply to twice the normal level.

Effect of pent-4-enoic acid and related fatty acids on blood ketone body levels

Pent-4-enoic acid and cyclopropanecarboxylic acid increased the total ketone body content of blood and reduced the β-hydroxybutyrate (βHB)/acetoacetate (AcAc) ratio in starved rats (Table 2). Pent-2-enoic and pentanoic acid increased the total ketone body content without changing the βHB/AcAc ratio significantly (Table 2).

The control levels of AcAc and βHB were similar to those found in starved rats by Berry, Williamson & Wilson (1965) and the effects of cyclopropanecarboxylic acid confirm the results of Stewart (1962) and of Williamson & Wilson (1965).

Effects of pent-4-enoic acid on plasma free fatty acid levels

There was an increase in the plasma free fatty acid level in fed rats to double the control value 40 min after injecting pent-4-enoic acid (175 mg/kg) (Table 3). The

Table 2. *Effects of pent-4-enoic acid and related fatty acids on blood ketone body levels.* Rats (200–250 g) were starved for 24 h before use to increase the total blood ketone body content up to 15 times normal. Starvation also stabilizes the β-hydroxybutyrate (βHB)/acetoacetate (AcAc) ratio at a higher value (Berry & others, 1965); this enables more accurate assay of these compounds. Blood was collected from the tail and 0·40 ml was added to 1·80 ml of 0·6 N $HClO_4$ to precipitate protein, and βHB and AcAc were assayed in the supernatant by the enzymic method of Williamson, Mellanby & Krebs (1962). The fatty acids were given intraperitoneally 20 min after the first blood sample was taken. The results are expressed as μmole of AcAc plus βHB/ml blood. Data are given for one experiment with each compound, with lower doses similar though smaller changes were obtained.

Control	Time	—20 min	1 h 10 min	3 h 5 min	4 h 10 min	6 h 30 min
0·4 ml	Total ketones	2·46	2·61	2·79	2·26	2·84
0·135 M NaCl	βHB/AcAc ratio	2·84	2·73	2·72	3·18	3·11
Pent-4-enoic acid	Time	—20 min	1 h 5 min	3 h	4 h 5 min	6 h 25 min
200 mg/kg	Total ketones	2·71	4·82	5·16	4·60	2·86
	βHB/AcAc ratio	2·82	1·00	0·91	0·92	2·32
Pent-2-enoic acid	Time	—20 min	1 h 15 min	3 h 15 min	4 h 15 min	6 h 15 min
150 mg/kg	Total ketones	2·56	3·16	3·11	2·72	2·90
	βHB/AcAc ratio	2·88	2·95	3·38	4·22	3·14
Pentanoic acid	Time	—20 min	65 min	3 h 5 min	4 h 6 min	6 h 35 min
300 mg/kg	Total ketones	2·56	4·20	4·15	4·00	3·20
	βHB/AcAc ratio	2·88	2·50	2·20	1·50	1·91
Cyclopropane-carboxylic	Time	—20 min	1 h	3 h	4 h	6 h
acid	Total ketones	2·59	4·40	5·60	5·81	3·10
150 mg/kg	βHB/AcAc ratio	3·05	1·44	1·17	1·00	1·82
Cyclobutane-carboxylic	Time	—20 min	55 min	2 h 55 min	3 h 55 min	6 h 55 min
acid,	Total ketones	2·56	3·16	3·55	3·66	2·70
150 mg/kg	βHB/AcAc ratio	2·88	2·68	2·74	2·86	2·37

Table 3. *Effect of pent-4-enoic acid on plasma free fatty acid levels.* The method of Duncombe (1963, 1964) was followed using rats (240–260 g) allowed a normal diet up to 4 h before the experiment. Pent-4-enoic acid or 0·135 M NaCl was given intraperitoneally at zero time. Animals were stunned at intervals by a blow on the head and 3 ml of blood was taken by cardiac puncture. The blood was transferred to ice-cold heparinized tubes, centrifuged at 3500 rev/min for 10 min and 0·5 ml of plasma used for analysis. Each result represents the free fatty acid levels in a single rat.

Injection	Time after injection (h min)	Free fatty acid level (mμmole/ml plasma)
0·14 M NaCl (0·50 ml)	0·00	256
	0·33	278
	1·05	224
	2·01	262
	2·34	240
Pent-4-enoic acid 175 mg/kg	0·25	262
	0·40	510
	1·00	510
	1·30	516
	1·50	430
	2·35	346
	3·00	280
	3·30	416
	4·00	334

control values were slightly lower than those given in the literature (De Renzo, McKerns & others, 1958; Hales & Kennedy, 1964).

Toxicities of pent-4-enoic acid and related fatty acids

The LD50 for pent-4-enoic acid in starved mice (315 mg/kg, Table 4) was similar to the value for starved rats (about 250 mg/kg) estimated from the data in Table 1. Most deaths occurred within the first hour. Cyclopropanecarboxylic acid (which is not hypoglycaemic in mice, Stewart, 1962) was more toxic than pent-4-enoic acid, but all the other fatty acids tested, including some not used elsewhere in this work, were less toxic (Table 4). The toxicity of pent-4-enoic acid was increased by starvation, the LD50 being even less after 22 h than after 18 h (Table 4). In contrast the toxicity of cyclopropanecarboxylic acid was unchanged after 22 h of starvation.

Table 4. *Toxicities of pent-4-enoic acid and related fatty acids in mice.* The LD50 values were estimated by the method of Weil (1952), using 6 animals per dose level, the 95% confidence limits are given by the figures in brackets. Mice (20–25 g) were allowed, unless otherwise stated, a normal diet before use. The ambient temperature was maintained at 21 ± 1° and water allowed freely. Fatty acid solutions were given intraperitoneally or subcutaneously (the toxicity was not influenced by the route of administration). Mice were pre-treated with riboflavin phosphate (12·5 mg/kg) and DL-carnitine (800 mg/kg) intraperitoneally 1 h before use where indicated. Deaths were scored after 24 h.

Fatty acid	LD50 (g/kg)
Pent-4-enoic acid	0·891 (0·665–1·19)
Pent-4-enoic acid	1·00 (0·733–1·14)
Pent-4-enoic acid*	0·871 (0·671–1·13)
Pent-4-enoic acid, pre-treated with riboflavin phosphate and DL-carnitine	0·794 (0·509–1·01)
Pent-4-enoic acid*, animals starved 18 h	0·575 (0·445–0·744)
Pent-4-enoic acid, animals starved 22 h	0·315 (0·237–0·437)
Pent-4-enoic acid, animals pre-treated with riboflavin phosphate and DL-carnitine, starved 22 h	0·315 (0·237–0·437)
Cyclopropanecarboxylic acid	0·172 (0·127–0·232)
Cyclopropanecarboxylic acid	0·223 (0·161–0·310)
Cyclopropanecarboxylic acid, animals pre-treated with riboflavin phosphate and DL-carnitine	0·159 (0·126–0·200)
Cyclopropanecarboxylic acid, animals starved for 22 h	0·230 (0·139–0·378)
Cyclopropanecarboxylic acid, animals starved for 22 h and pre-treated with riboflavin phosphate and DL-carnitine	0·223 (0·161–0·310)
Acrylic acid	1·59 (1·26–2·00)
Crotonic acid	3·59 (2·62–4·50)
Pent-2-enoic acid	1·58 (1·26–2·00)
Sorbic acid	2·82 (2·45–3·24)
Hex-3-enoic acid	1·84 (1·29–2·20)
Hept-2-enoic acid	1·60 (1·05–2·41)
Butyric acid	3·18 (2·51–4·05)
Pentanoic acid	3·59 (2·62–4·50)
Hexanoic acid	3·18 (2·51–4·05)
Cyclobutanecarboxylic acid	1·27 (1·00–1·61)

*Estimations using 10 animals per dose level.

These figures may be compared with those for hypoglycin. Entman & Bressler (1967) gave doses of 500–750 mg/kg to mice starved for 18 h and did not report any deaths. The LD50 in fed rats was about 100 mg/kg and fasting halved this value (Feng & Patrick, 1958; Hassall & Reyle, 1955a, b). Hypoglycin therefore appears to be more toxic than pent-4-enoic acids in rats but not in mice. Hypoglycin is very toxic in rabbits (toxic dose 10–20 mg/kg; Chen, Anderson & others, 1957).

Holt & Holt (1959) reported that feeding riboflavin phosphate to rats and mice antagonized the toxicity and hypoglycaemic effects of hypoglycin. Entman & Bressler (1967) found that L-carnitine (12 mg/mouse; 360–600/mg/kg) given intravenously with hypoglycin also antagonized the hypoglycaemic effects. Attempts were therefore made to modify the toxicity of pent-4-enoic acid by intraperitoneal administration of these two unrelated compounds (though it is appreciated that there may be no simple relation between toxicity and hypoglycaemia). Pre-treatment with riboflavin phosphate (12·5 mg/kg) and DL-carnitine (800 mg/kg) had no significant effect on the toxicity of pent-4-enoic acid in fed or in starved mice (Table 4). They also had no effect when given at the same time as pent-4-enoic acid; or when given in divided doses one h before and at the same time as pent-4-enoic acid. Neither riboflavin phosphate or DL-carnitine given separately modified toxicity. Free riboflavin (12·5 mg/kg) also did not change the toxicity. In control experiments riboflavin phosphate plus DL-carnitine had no apparent effect when given to mice. These compounds had no effect on the toxicity of cyclopropanecarboxylic acid in fed or in starved mice (Table 4).

DISCUSSION

The maximum hypoglycaemic effect of pent-4-enoic acid is seen about 2 h after administration in the rat and after 30 min in the mouse. With hypoglycin the maximum effect in the rat is after 4–6 h (Feng & Patrick, 1958) and in the mouse after about 90 min (Entman & Bressler, 1967). This is consistent with the view that pent-4-enoic acid is active without further metabolism, but that hypoglycin must first be converted *in vivo* into methylenecyclopropaneacetic acid (Holt, 1966). None of the four structurally related fatty acids used here was hypoglycaemic in rats although cyclopropanecarboxylic acid causes hypoglycaemia in the guinea-pig and monkey by a mechanism which depends on circulating insulin (Stewart, 1962). No explanation can be offered for the lack of effect of pent-4-enoic acid on blood glucose levels in the rabbit, though hypoglycin is also not hypoglycaemic in some species (Chen & others, 1957).

Evidence that pent-4-enoic acid and hypoglycin do not lower blood sugar levels by influencing insulin secretion or activity may be summarized as follows. Blood glucose does not fall until liver glycogen is depleted (Patrick, 1954). Insulin reduces serum free fatty acid levels, whilst pent-4-enoic acid and hypoglycin increase them (McKerns & others, 1960). Infusion of ketone-bodies causes hypoglycaemia in dogs, most probably by stimulating insulin secretion (Mebane & Madison, 1964) although ketone-bodies also reduce glucose utilization since βHB and AcAc are preferred fuels in most tissues (Randle, Newsholme & Garland, 1964). Large amounts of AcAc given parenterally to rats produced small falls in blood glucose, though blood levels of AcAc reached during starvation were not hypoglycaemic (Tidwell & Axelrod, 1948). Both cyclopropanecarboxylic acid and pentanoic acid caused an increase in total ketone bodies similar to those caused by pent-4-enoic acid or by hypoglycin (Williamson & Wilson, 1965), yet neither was hypoglycaemic. It is therefore unlikely that ketosis contributes to the hypoglycaemic effects of pent-4-enoic acid or hypoglycin (cf. Chen & others, 1957). Lowered glucagon secretion may conceivably cause hypoglycaemia but Chen & others (1957), Leppla & Holt (1956) and Feng (1957) found no evidence for impaired glucagon secretion after hypoglycin treatment. Hypoglycin did not alter secretion of adrenal glucocorticoids (McKerns & others, 1960).

Both methylenecyclopropaneacetic acid (Holt, Holt & Böhm, 1966) and pent-4-enoic acid (Senior & Sherratt, 1967; Senior & others, 1968) strongly inhibit the

oxidation of long-chain fatty acids. This could account for raised levels of serum free fatty acids found in hypoglycin-treated rats (De Renzo & others, 1958) and in pent-4-enoic acid-treated rats.

Pent-2-enoic acid and pentanoic acid caused an increase in the total blood ketone body levels in mice without altering the βHB/AcAc ratio. Pent-4-enoic acid, and also hypoglycin and cyclopropanecarboxylic acid (Stewart, 1962; Williamson & Wilson, 1965), increased the total ketone body levels and reduced the βHB/AcAc ratio. A reduction of the blood βHB/AcAc ratio reflects reduction in the NADH/NAD^+ ratio within mitochondria (Berry & others, 1965). This cannot be related simply to inhibition of fatty acid oxidation as suggested by Williamson & Wilson (1965) since cyclopropanecarboxylic acid does not inhibit this process (Senior & others, 1968). The increase in total blood ketone bodies is probably due to greater impairment of their peripheral utilization than of their formation by the liver following administration of these three fatty acids (Williamson & Wilson, 1965; Senior & others, 1968).

Glucose utilization in hypoglycin-treated animals is either reduced or unchanged while palmitate or stearate utilization is strongly inhibited (McKerns & others, 1960; Holt & others, 1966). Holt & others (1966) suggested that gluconeogenesis may be inhibited following hypoglycin administration. We have shown that pent-4-enoic acid, but none of the four control fatty acids, strongly inhibits gluconeogenesis *in vitro* (Senior & Sherratt, 1968b). The most probable explanation of the hypoglycaemic effects of pent-4-enoic acid and of hypoglycin, therefore, is that when long-chain fatty acid and ketone body oxidation are blocked the only available major fuel is glucose. Glygocen reserves become exhausted and since glucose cannot be replaced by gluconeogenesis hypoglycaemia ensues (Senior, 1967). The greater toxicity of pent-4-enoic acid and of hypoglycin (Feng & Patrick, 1958) but not of cyclopropanecarboxylic acid, in starved than in fed animals agrees with this interpretation.

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REFERENCES

ANDERSON, H. W., JOHNSON, J. L., NELSON, J. W., OLSON, E. C., SPEETER, M. E. & VAVRA, J. J. (1958). *Chemy Ind.*, 330.

BERRY, M. N., WILLIAMSON, D. H. & WILSON, M. B. (1965). *Biochem. J.*, **94**, 17–19c.

CHEN, K. K., ANDERSON, R. C., MCCOWEN, M. C. & HARRIS, P. N. (1957). *J. Pharmac. exp. Ther.*, **121**, 272–285.

DE RENZO, E. C., MCKERNS, K. W., BIRD, H. H., CEKLENIAK, W. P., COULOMB, B. & KALEITA, E. (1958). *Biochem. Pharmac.*, **1**, 236–237.

DUNCOMBE, W. G. (1963). *Biochem. J.*, **88**, 7. 10.

DUNCOMBE, W. G. (1964). *Clinica chim. Acta*, **9**, 122–125.

ENTMAN, N. & BRESSLER, R. (1967). *Molec. Pharmac.*, **3**, 333–340.

FENG, P. C. (1957). *Nature, Lond.*, **180**, 855–856.

FENG, P. C. & PATRICK, S. J. (1958). *Br. J. Pharmac. Chemother.*, **13**, 125–130.

HALES, C. N. & KENNEDY, G. C. (1964). *Biochem. J.*, **90**, 620–624.

HASSALL, C. H. & REYLE, K. (1955a). *West Indian Med. J.*, **4**, 83–90.

HASSALL, C. H. & REYLE, K. (1955b). *Biochem. J.*, **60**, 334–339.

HASSALL, C. H., REYLE, K. & FENG, P. C. (1954). *Nature, Lond.*, **173**, 356–357.

HOLT, C. VON (1966). *Biochem. biophys. Acta*, **125**, 1–10.

HOLT, L. VON & HOLT, C. VON (1959). *Biochem. Z.*, **331**, 422–429.

HOLT, C. VON, HOLT, M. VON & BÖHM, H. (1966). *Biochem. biophys. Acta*, **125**, 11–21.

LEPPLA, W. & HOLT, C. VON (1956). *Arch. exp. Path. Pharmac.*, **228**, 166–167.

MEBANE, D. & MADISON, L. L. (1964). *J. Lab. clin. Med.*, **63**, 177–192.

MCKERNS, K. W., BIRD, H. H., KALEITA, E., COULOMB, B. S. & DE RENZO, E. C. (1960). *Biochem. Pharmac.*, **3**, 305–315.

PATRICK, S. J. (1954). *J. appl. Physiol.*, **7**, 140–142.

RANDLE, P. J., NEWSHOLME, E. A. & GARLAND, P. B. (1964). *Biochem. J.*, **93**, 652–665.

SAMPSON, F. E. & DAHL, N. (1955). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **14**, 129.

SAMPSON, F. E., DAHL, N. & DAHL, D. R. (1956). *J. clin. Invest.*, **35**, 1291–1298.

SENIOR, A. E. (1967). Ph.D. Dissertation, University of Newcastle upon Tyne.

SENIOR, A. E. & SHERRATT, H. S. A. (1966). *Biochem. J.*, **100**, 71P.

SENIOR, A. E. & SHERRATT, H. S. A. (1967). *Ibid.*, **104**, 56P.

SENIOR, A. E. & SHERRATT, H. S. A. (1968a). *Ibid.*, **110**, 499–509.

SENIOR, A. E. & SHERRATT, H. S. A. (1968b). *Ibid.*, **110**, 521–527.

SENIOR, A. E., ROBSON, B. & SHERRATT, H. S. A. (1968). *Ibid.*, **110**, 511–519.

STEWART, G. A. (1962). *Anglo-German Med. Rev.*, **1**, 334–347.

TIDWELL, H. C. & AXELROD, H. E. (1948). *J. biol. Chem.*, **172**, 179–184.

WEIL, C. (1952). *Biometrics*, **8**, 249–263.

WILLIAMSON, D. H., MELLANBY, J. & KREBS, H. A. (1962). *Biochem. J.*, **82**, 90–96.

WILLIAMSON, D. H. & WILSON, M. B. (1965). *Ibid.*, **94**, 19–20c.