Biochemical Pharmacology, Vol. 21, pp. 3153–3162. Pergamon Press, 1972. Printed in Great Britain.

EFFECTS OF METFORMIN ON GLUCOSE UPTAKE BY ISOLATED DIAPHRAGM FROM NORMAL AND DIABETIC RATS

K. N. FRAYN and P. I. ADNITT

Department of Clinical Pharmacology and Diabetic Clinic,
St. Bartholomew's Hospital, London, E.C.1, England

(*Received* 24 *April* 1972; *accepted* 6 *July* 1972)

Abstract—The effects of the antidiabetic drug metformin (NI, NI-dimethylbiguanide) on glucose uptake by isolated rat diaphragm muscle have been studied. A therapeutic concentration of metformin (10 μg/ml) had no effect on glucose uptake by diaphragm muscle from normal rats incubated in the absence or presence of insulin (100 and 1000 μU/ml), but increased uptake by diaphragm muscle from alloxan-diabetic rats incubated in the presence of insulin ($P < 0.05$). Diaphragm muscle from normal rats incubated in a medium containing sodium butyrate (0·25 mg/ml) showed a reduction in glucose uptake similar to that seen in muscle from diabetic animals. Metformin (10 μg/ml) also increased glucose uptake by this preparation in the presence of insulin ($P < 0.01$). A higher concentration of metformin (100 μg/ml) caused a depression of glucose uptake by diaphragms from normal rats, and the necessity for studying therapeutic concentrations of the biguanide drugs is stressed. The relation of these findings to the antidiabetic effect of the drug in man is discussed. The mechanisms involved are discussed in terms of changes in glycogen metabolism.

THE BIGUANIDE antidiabetic drugs have a variety of metabolic effects *in vitro*, but the relevance of these effects to the therapeutic action of the drugs is unclear. These effects include inhibition of gluconeogenesis from a number of substrates in perfused[1,2] and minced[3] liver preparations from rat and guinea-pig and in rat kidney slices,[4,5] inhibition of glucose uptake by rat[6] and hamster[7,8] small intestine and inhibition of oxidation of glucose, pyruvate and acetate in a number of tissues.[3,9] These effects may all be related[3] to the inhibition of oxidative phosphorylation produced by phenformin *in vitro*.[10,11] However, in diabetic patients treated with phenformin glucose oxidation may in fact be increased.[12] The elevation of fasting blood lactic acid levels seen in some patients treated with phenformin[13–16] suggests that this drug may produce inhibition of aerobic oxidation *in vivo*, but this is not a necessary part of the antidiabetic action since some patients respond to biguanide treatment without rise in blood lactic acid levels,[17] and metformin does not in any case produce a rise in blood lactic acid when used to control diabetes.[18]

A major difficulty in interpreting the results of *in vitro* studies of the biguanide drugs is that the metabolic effects of these drugs may be related to their concentration. Phenformin and metformin, for instance, inhibit glucose absorption by everted rat jejunum when present in concentrations greater than 10^{-3} M (about 200 and 150 μg/ml, respectively), but cause increased absorption of glucose when present at lower concentrations.[19] Buformin in concentrations similar to its therapeutic plasma level increases glucose oxidation in rat adipose tissue *in vitro*, but this effect diminishes as the drug concentration is raised.[20] The stimulation of glucose uptake into rat adipose

tissue *in vitro* seen at a low concentration of phenformin (5 μg/ml) occurs without increased lactate production, but as the drug concentration is raised an increase in lactate output is seen.[21]

One action of the biguanide drugs *in vivo* may be to increase glucose uptake into skeletal muscle. Phenformin treatment increases glucose uptake in the human forearm in diabetic patients, and not in non-diabetic subjects,[22–24] and an increase in peripheral glucose disposal may account for the stimulation of Cori cycle activity seen when diabetic patients are treated with phenformin.[12] Similarly, the clearance of intravenously-administered glucose is significantly speeded up in diabetic patients after treatment with metformin.[25] A number of studies have been made on the effects of the biguanide drugs on glucose uptake by the isolated rat diaphragm preparation, but with conflicting results. Again this is probably because a wide range of drug concentrations have been used. High concentrations of phenformin added to diaphragms from normal rats cause increased glucose uptake and depression of glycogen content,[26–29] this effect being independent of exogenous insulin,[30] and a similar effect is seen with diaphragms from alloxan-diabetic rats incubated with phenformin at 1 mg/ml.[29] However, at lower concentrations of phenformin the increased glucose uptake is dependent on the presence of exogenous insulin,[30] and with buformin in low concentrations diaphragms incubated in human serum (containing insulin-like activity) show depression of uptake.[20] In addition, any explanation of the mode of action of the biguanide drugs must be able to account for their lack of hypoglycaemic effect in non-diabetic individuals under normal circumstances, and yet there have been no satisfactory studies comparing the effects of the drugs *in vitro* on glucose uptake by muscle from normal and diabetic animals.

The experiments to be described were therefore designed to test the effects of metformin, in a concentration comparable with its therapeutic blood level, on glucose uptake by isolated rat diaphragm, using tissue from both normal and diabetic rats.

EXPERIMENTAL

Animals. Wistar rats of either sex initially weighing 100–130 g were used. All rats were fasted for 24 hr before killing.

Preparation of alloxan-diabetic rats. Diabetes was induced by administration of a freshly-prepared solution of alloxan in water (250 mg alloxan anhydrate/kilogram body wt) intraperitoneally. Rats were used 48–96 hr after injection, having been fasted for 24 hr before killing. Blood was taken after killing but before removal of the diaphragm for estimation of the fasting blood glucose level. Rats used in the study all had fasting blood glucose levels greater than 120 mg/100 ml (normal value under these conditions: 55 $\pm$ 10 mg/100 ml, mean $\pm$ S.D., N = 21).

Reagents. Metformin hydrochloride was a gift from Rona Laboratories Ltd., Hitchin. All concentrations of metformin are expressed in terms of the hydrochloride. Recrystallised ox insulin (24·0 U/mg) was a gift from Burroughs Wellcome and Co., Dartford. Bovine serum albumin (Cohn fraction V) and oyster glycogen were from Sigma (London) Chemical Co., Ltd. Reagents for the glucose oxidase technique were from Hughes and Hughes (Enzymes) Ltd., Brentwood. All other reagents were of analytical grade.

Preparation and incubation of diaphragms. The incubation medium was Krebs–Henseleit bicarbonate buffer[31] saturated with 95% oxygen, 5% carbon dioxide, to

which was added bovine serum albumin (2 mg/ml), to prevent losses of insulin by adsorption to glass,[32] and glucose (3 mg/ml). Metformin hydrochloride and sodium butyrate were dissolved in this medium as required. Insulin solutions were prepared by dilution with the medium from a stock solution (1 mg/ml) in 0·03 M–HCl.

Hemidiaphragms were prepared by the method of Vallance-Owen and Hurlock.[33] They were rinsed for 15 min in ice-cold medium with no additions, then gently blotted, weighed on a torsion balance, and placed in a conical flask containing 2 ml of medium with the appropriate additions. The flasks were incubated with shaking at 37° for 90 min. The hemidiaphragms were then blotted and dissolved in 2 ml 30% (w/v) KOH at 100° for estimation of glycogen content. Duplicate samples of residual medium were taken for glucose estimation.

Glucose and glycogen estimation. Glucose was estimated by a glucose oxidase technique.[34] Glycogen was estimated by a method based on that of Walaas and Walaas.[35] After hydrolysis of glycogen the resulting glucose was estimated by the glucose oxidase method as above.

Experimental design. One hemidiaphragm from each rat was used as a control (no metformin added). The other was incubated under identical conditions but with metformin added. Equal numbers of left and right hemidiaphragms were used as controls. Experimental design was balanced to eliminate any effects resulting from the order of commencing incubation.

Results are expressed as amount of glucose utilized during 90 min (or as glycogen content after incubation) in milligrams per gram wet wt of tissue. Percentage changes due to drug are calculated "within rats", and mean percentage changes are shown for each experiment.

RESULTS

Effects of metformin on normal isolated rat diaphragm. Metformin in a concentration of 10 μg/ml had no significant effects on glucose uptake by diaphragm muscle from non-diabetic rats in the presence of various concentrations of insulin. At a concentration of 100 μg/m metformin caused a significant depression of uptake (Fig. 1).

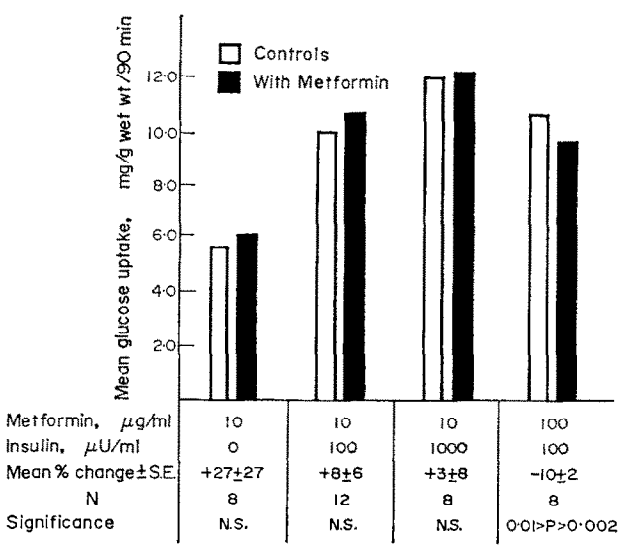

FIG. 1. Effects of metformin on glucose uptake by isolated hemidiaphragms from non-diabetic rats.

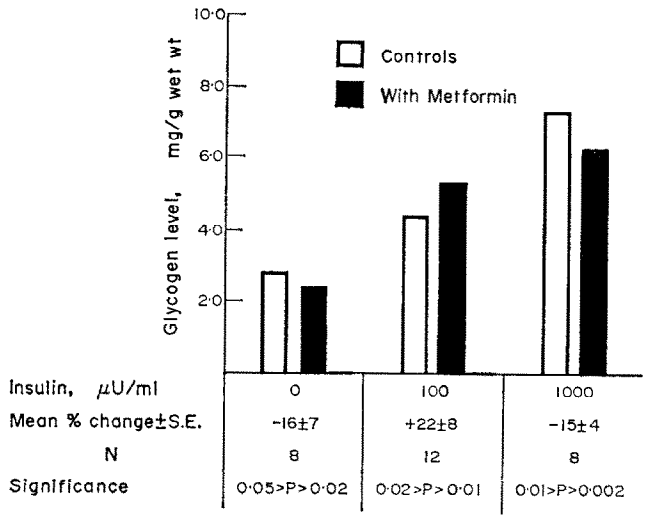

FIG. 2. Effects of metformin (10 μg/ml) on glycogen content (after 90 min incubation) of isolated hemidiaphragms from non-diabetic rats.

The effects of metformin (10 μg/ml) on glycogen deposition by this preparation are shown in Fig. 2. The glycogen content of the muscle was decreased after incubation with metformin in the absence of insulin and in the presence of a high insulin concentration (1000 μU/ml), and increased by metformin in the presence of insulin at an intermediate concentration of 100 μU/ml.

Effects of metformin on diaphragms from alloxan-diabetic rats. Glucose uptake by diaphragms from alloxan-diabetic rats was significantly reduced both in the absence

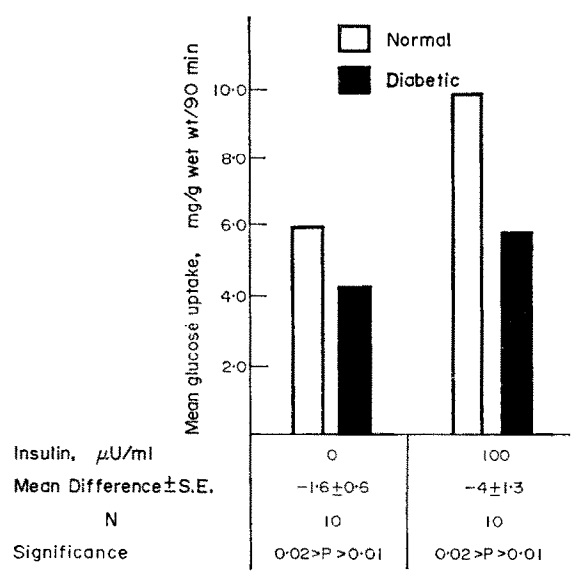

FIG. 3. Comparison of glucose uptake by isolated hemidiaphragms from normal and matched alloxan-diabetic rats.

and the presence of insulin (100 μU/ml) when compared on a "between rats" basis with diaphragms from normal rats, matched with respect to the time of killing (Fig. 3). The percentage increase in uptake due to insulin (100 μU/ml) was also reduced in these diaphragms. (Percentage increase in glucose uptake caused by insulin (between-rats comparisons): Diabetic + 37 $\pm$ 32 per cent, Normal + 65 $\pm$ 14 per cent, means $\pm$ S. E.; N = 10 in each case.) Metformin (10 μg/ml) caused a significant increase in glucose uptake by diaphragms from alloxan-diabetic rats in the presence of insulin (100 μU/ml) but had no significant effect in the absence of insulin (Fig. 4). Changes in glycogen level produced by metformin were not significant at the 0·05 level in either case. (Results not shown.)

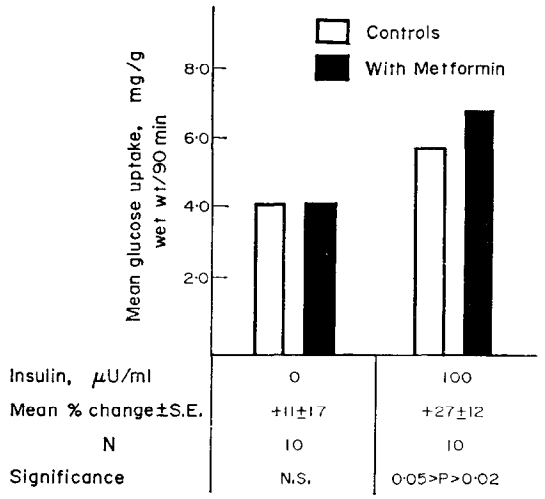

FIG. 4. Effects of metformin (10 μg/ml) on glucose uptake by isolated hemidiaphragms from alloxan-diabetic rats.

Effects of metformin on diaphragms incubated with free fatty acids (FFA). Diaphragms from normal rats incubated in a medium containing sodium *n*-butyrate (0·25 mg/ml) show reduced glucose uptake.[36,37] In the present experiments this reduction occurred both in the absence and presence of insulin (100 μU/ml) but was statistically significant only in the latter case (Fig. 5). Metformin (10 μg/ml) caused a significant increase in glucose uptake by this preparation in the presence of insulin (100 μU/ml) and a significant depression of uptake in the absence of insulin (Fig. 6). Glycogen levels were increased and decreased respectively by metformin, but in neither case was the change significant at the 0·05 level. (Results not shown.)

DISCUSSION

An important feature of the biguanide drugs is their lack of hypoglycaemic effect in normally-fed non-diabetic individuals. In the present study it was shown that metformin in a therapeutic concentration of 10 μg/ml has no effect on glucose uptake by diaphragm muscle from non-diabetic rats in the presence of various concentrations of insulin, but causes a significant increase in glucose uptake by diaphragms from alloxan-diabetic rats and by normal diaphragms incubated with sodium butyrate in

the presence of insulin at a physiological concentration (100 μU/ml). The metabolism of diaphragms incubated with a high concentration of FFA is similar to that seen in diabetes, as will be described below. These results are therefore consistent with the effects of the drug in man.

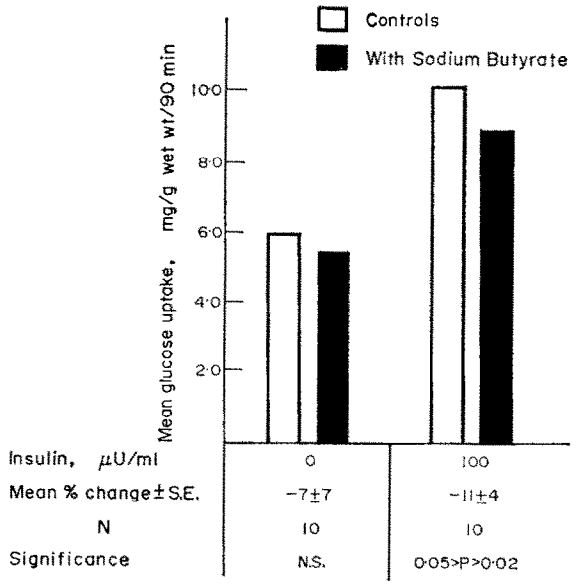

FIG. 5. Effects of sodium *n*-butyrate (0·25 mg/ml) on glucose uptake by isolated hemidiaphragms from non-diabetic rats.

These results are in conflict with those of previous studies showing that phenformin and buformin may increase uptake by diaphragms from non-diabetic rats. As discussed above this may be due to the use in earlier experiments of high drug concentrations. The therapeutic blood levels of phenformin and buformin in man are of the order of 0·2 μg/ml.[38,39] Most previous studies have involved concentrations of phenformin in the range 120–1000 μg/ml,[26–29] although Daweke and Bach[20] showed that buformin in a concentration of 10 μg/ml causes increased uptake by normal rat diaphragm incubated in buffer without added insulin. In the present study it was shown that a concentration of metformin of 100 μg/ml, ten times greater than its maximum therapeutic blood level in man,[40] produced a depression of glucose uptake by normal rat diaphragm, and this unexpected result emphasises the danger with the biguanide drugs of extrapolation from results obtained with high drug concentrations.

The distinction between the effects of the drug at its therapeutic concentration on glucose uptake by diaphragms from normal and diabetic animals is explicable by considering the metabolic differences between these tissues. In normal rat heart and diaphragm membrane transport of glucose is the major rate-limiting step in glucose utilization.[41–44] In muscle from a diabetic animal the membrane transport of glucose is reduced when compared with normal muscle, and this defect is not fully overcome by addition of insulin *in vitro*.[45–48] Under these conditions free intracellular glucose

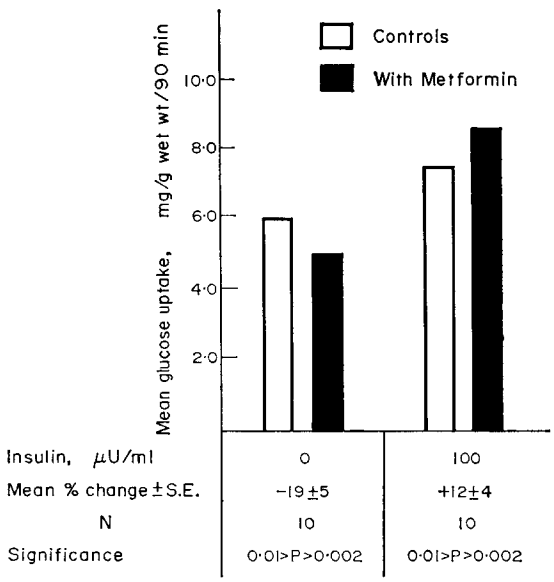

FIG. 6. Effects of metformin (10 μg/ml) on glucose uptake by isolated hemidiaphragms from non-diabetic rats in the presence of sodium *n*-butyrate (0·25 mg/ml).

accumulates,[46,49,50] showing that the phosphorylation of glucose by hexokinase (EC 2.7.1.1) is also impaired and may become the rate-limiting step in glucose utilization in muscle from a diabetic animal. This impairment of glucose uptake and insulin sensitivity in diabetes, which has been demonstrated with the perfused rat heart and "intact" diaphragm preparations, was confirmed in the present study using the isolated rat hemidiaphragm (Fig. 3).

The depression of hexokinase activity in diabetes is due to a decrease in the total activity of this enzyme[51] as well as to regulation of its activity by the intracellular concentration of glucose 6-phosphate (Glc 6-P), which is known to be a non-competitive inhibitor *in vitro*.[52] The abnormal FFA metabolism associated with diabetes[53–55] may be involved.[36,37] Many of the metabolic changes associated with diabetes, including the depression of hexokinase activity, are reproduced when normal heart or diaphragm muscle is perfused with, or incubated in, a medium containing fatty acids or ketone bodies.[48,56] In the present study sodium *n*-butyrate (0·25 mg/ml) was shown to impair glucose uptake by isolated rat diaphragm (Fig. 5). Short-chain fatty acids such as butyric produce more consistent inhibition of glucose uptake than do long-chain acids, probably because of the removal of the latter for esterification at high insulin concentrations.[37] The elevation of intracellular acetyl-CoA concentration caused by fatty acid oxidation leads, via an increase in the citrate level, to inhibition of phosphofructokinase (EC 2.7.1.11) and hence to an increased concentration of Glc 6-P.[48] In normal muscle in which membrane transport of glucose is the rate-limiting step in its uptake, regulation of hexokinase by the intracellular Glc 6-P concentration does not control membrane transport of monosaccharides.[57]

The stimulation of glucose uptake by metformin is thus seen in cases where the phosphorylation of glucose may be rate-limiting. This is exactly the result to be ex-

pected if metformin, in the presence of insulin, causes an increase in the rate of utilization of Glc 6-P. In normal muscle, in which membrane transport of glucose is the rate-limiting step, uptake of glucose is not affected, but when muscle from diabetic animals or normal muscle incubated with FFA is stimulated with insulin so that phosphorylation becomes rate-limiting, a reduction in Glc 6-P level will relieve inhibition of hexokinase, and the resultant drop in intracellular free glucose concentration will cause increased membrane transport of glucose.

Some clues to the site of action of metformin in the muscle cell are provided by the observations of the changes in glycogen deposition produced by metformin in normal muscle (Fig. 2). In the presence of insulin at a physiological concentration (100 μU/ml) the glycogen content is increased by metformin (10 μg/ml). This action of metformin ("Action 1"), a potentiation of the effect of insulin on glycogen synthesis, will result in an increased rate of removal of Glc 6-P just as is necessary to explain the effects of metformin on uptake by the "diabetic" muscle preparations.

The depression of glycogen content seen in the absence of insulin, and in the presence of a high concentration of insulin (1000 μU/ml) shows that metformin has an additional effect (Action 2) opposed to that described above, this latter action being independent of insulin.

The stimulation of glycogen synthesis by insulin is mediated by an increase in the proportion of the "I" (physiologically active[58]) form of the enzyme glycogen synthetase (EC 2.4.1.11).[59] These two actions of metformin may thus be seen as an inhibitory action independent of insulin (Action 2), and a potentiation of the effect of insulin on the conversion of the "D" (physiologically inactive[58]) to the "I" form of glycogen synthetase (Action 1). Since the insulin-stimulation of glycogen synthesis in this preparation is fully saturated at a concentration of insulin of 1000 μU/ml (unpublished observations), the inhibitory action (2) is again seen at high insulin concentrations.

This leads to the prediction that in the absence of exogenous insulin the intracellular Glc 6-P concentration will be raised by metformin, and that membrane transport of glucose would be depressed in a situation in which phosphorylation is rate-limiting. This is confirmed by the depression of glucose uptake caused by metformin in normal diaphragms incubated with butyrate in the absence of added insulin (Fig. 6). No significant effect is seen in diaphragms from diabetic rats in the absence of insulin (Fig. 4) in which membrane transport is probably so reduced by the lack of endogenous insulin that it is itself rate-limiting.

Previous workers have shown separate insulin–dependent and insulin–independent actions of biguanides on glucose uptake into rat diaphragm muscle[30] and adipose tissue.[21] This hypothesis may be simplified by a further speculation. Conversion of the "D" to the "I" form of glycogen synthetase is mediated by the enzyme synthetase "D" phosphatase, which is subject to product inhibition by glycogen both *in vitro*[60] and *in vivo*.[59] Both the postulated actions of metformin may be explained by suggesting that the drug is bound preferentially to the glycogen binding site, producing some degree of inhibition of this enzyme (Action 2). On stimulation of glycogen synthesis by insulin the normal feedback inhibition by glycogen would be prevented, and so a potentiation of the insulin effect would be seen (Action 1).

Whether or not these effects occur during biguanide treatment *in vivo* remains to be tested, and information on glycogen levels in diabetic patients or animals treated

with these drugs would be valuable. In rats, chronic treatment with phenformin (100 mg/kg body wt s.c. for 7 days) produces a marked increase in the glycogen content of the diaphragm, heart and liver when compered with saline-treated controls.[61] No information is available in the case of metformin.

Acknowledgements—We would like to thank Rona Laboratories Ltd., and the Board of Governers, St. Bartholomew's Hospital, for financial support. Also Drs. K. O. Black and P. Turner for their advice and encouragement.

REFERENCES

1. R. A. Altschuld and F. A. Kruger, *Ann. N.Y. Acad. Sci.* **148,** 612 (1968).
2. J. J. Connon, *European Association for the Study of Diabetes*, 7th Annual Meeting, Southampton, Abstract 42, Novo, Copenhagen (1971).
3. N. O. Jangaard, J. N. Pereira and E. R. Pinson, *Diabetes* **17,** 96 (1968).
4. F. Meyer, M. I. Ipaktchi and H. Clauser, *Nature, Lond.* **213,** 203 (1967).
5. B. Lescure and P. Volfin, *Biochimie* **53,** 391 (1971).
6. E. Lorch, *Diabetologia* **7,** 195 (1971).
7. W. Creutzfeldt, B. Willms and W. Caspary, *Reports on Oral Diabetes Therapy*, A selection of papers presented at the 7th Congress of the International Diabetes Federation, Buenos Aires, 1970, p. 95, Excerpta Medica, Amsterdam (1971).
8. W. Caspary, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **269,** 421 (1971).
9. J. N. Pereira, N. O. Jangaard and E. R. Pinson, *Diabetes* **16,** 869 (1967).
10. A. N. Wick, E. R. Larson and G. S. Serif, *J. biol. Chem.* **233,** 296 (1958).
11. A. B. Falcone, R. L. Mao and E. Shrago, *J. biol. Chem.* **237,** 904 (1962).
12. G. L. Searle and R. R. Cavalieri, *Ann. N.Y. Acad. Sci.* **148,** 734 (1968).
13. J. A. Moorhouse, S. S. Fajans and J. W. Conn, *Clin. Res.* **6,** 405 (1958).
14. J. W. Craig, M. Miller, H. Woodward and E. Merik, *Diabetes* **9,** 186 (1960).
15. R. S. Walker, A. L. Linton and W. J. T. Thomson, *Br. med. J.* **2,** 1567 (1960).
16. S. K. Varma, S. J. Heaney, W. G. Whyte and R. S. Walker, *Br. med. J.* **1,** 193 (1972).
17. R. S. Walker, 7th Congress of the International Diabetes Federation, Buenos Aires, *Excerpta med.* International Congress Series, No. 209, p. 95 (1970).
18. G. Debry, A. Cherrier and J. Laurent, *Diabète* **12,** 239 (1964).
19. A. H. G. Love, *Diabetologia* **5,** 422 (1969).
20. H. Daweke and I. Bach, *Metabolism* **12,** 319 (1963).
21. R. E. Tranquada and P. M. Beigelman, *Clin. Res.* **8,** 248 (1960).
22. W. J. H. Butterfield, *Ann. N.Y. Acad. Sci.* **148,** 724 (1968).
23. W. J. H. Butterfield and M. J. Wichelow, *Lancet* **2,** 785 (1968).
24. M. J. Wichelow and W. J. H. Butterfield, *Postgrad. med. J.* May Suppl. 24 (1969).
25. K. N. Frayn, P. I. Adnitt and P. Turner, *Postgrad. med. J.* **47,** 777 (1971).
26. R. H. Williams, J. M. Tyberghein, P. M. Hyde and R. L. Nielsen, *Metabolism* **6,** 311 (1957).
27. O. J. Rafaelsen, *Metabolism* **8,** 195 (1959).
28. P. H. Wright, *Biochem. J.* **71,** 633 (1959).
29. D. W. Clarke and N. Forbath, *Diabetes* **9,** 167 (1960).
30. R. E. Bolinger, W. P. McKee and J. W. Davis, *Metabolism* **9,** 30 (1960).
31. R. M. C. Dawson, D. C. Elliott, W. H. Elliott and K. M. Jones (Eds.) in *Data for Biochemical Research*), p. 507, Clarendon Press, Oxford (1969).
32. P. H. Sönksen, J. P. Ellis, C. Lowy, A. Rutherford and J. D. N. Nabarro, *Diabetologia* **1,** 208 (1965).
33. J. Vallance-Owen and B. Hurlock, *Lancet* **1,** 68 (1954).
34. G. Morley, A. Dawson and V. Marks, *Proc. Ass. clin. Biochim.* **5,** 42 (1968).
35. O. Walaas and E. Walaas, *J. biol. Chem.* **187,** 769 (1950).
36. P. J. Randle, P. B. Garland, C. N. Hales and E. A. Newsholme, *Lancet* **1,** 785 (1963).
37. P. J. Randle, P. B. Garland, C. N. Hales, E. A. Newsholme, R. M. Denton and C. I. Pogson, *Recent Prog. Horm. Res.* **22,** 1 (1966).
38. R. Beckmann, *Ann. N.Y. Acad. Sci.* **148,** 820 (1968).
39. R. Beckmann, W. Lintz and E. Schmidt-Böthelt, in 7th Congress of the International Diabetes Federation, Buenos Aires, *Excerpta med.* International Congress Series, No. 209, p. 84 (1970).
40. P. Pignard, *Ann. Biol. clin.* **20,** 325 (1962).
41. C. R. Park, J. Bornstein and R. L. Post, *Am. J. Physiol.* **182,** 12 (1955).
42. D. M. Kipnis, *Ann. N.Y. Acad. Sci.* **82,** 354 (1959).
43. J. R. Neely, H. Liebermeister and H. E. Morgan, *Am. J. Physiol.* **212,** 815 (1967).

44. L. H. OPIE, K. R. L. MANSFORD and P. OWEN, *Biochem. J.* **124,** 475 (1971).
45. H. E. MORGAN, M. J. HENDERSON, D. M. REGEN and C. R. PARK, *Ann. N.Y. Acad. Sci.* **82,** 387 (1959).
46. C. R. PARK and H. E. MORGAN, *Diabetes* **9,** 250 (1960).
47. F. A. RIDDICK, D. M. REISLER and D. M. KIPNIS, *Diabetes* **11,** 171 (1962).
48. P. J. RANDLE, E. A. NEWSHOLME and P. B. GARLAND, *Biochem. J.* **93,** 652 (1964).
49. H. E. MORGAN and C. R. PARK, *J. clin. Invest.* **36,** 916 (1957).
50. D. M. KIPNIS and C. F. CORI, *J. biol. Chem.* **235,** 3070 (1960).
51. I. DAS, *Biochem. J.* **127,** 22p (1972).
52. R. K. CRANE and A. SOLS, *J. biol. Chem.* **203,** 273 (1953).
53. E. L. BIERMAN, V. P. DOLE and T. N. ROBERTS, *Diabetes* **6,** 475 (1957).
54. L. A. CARLSON and J. ÖSTMAN, *Acta med. Scand.* **174,** 215 (1963).
55. M. MATTOCK, A. CHAIT, M. MANCINI and B. LEWIS, *Clin. Sci.* **42,** 18p (1972).
56. P. B. GARLAND and P. J. RANDLE, *Biochem. J.* **93,** 678 (1964).
57. E. A. NEWSHOLME and P. J. RANDLE, *Biochem. J.* **80,** 655 (1961).
58. R. PIRAS, L. B. ROTHMAN and E. CABIB, *Biochemistry* **7,** 56 (1968).
59. W. H. DANFORTH, *J. biol. Chem.* **240,** 588 (1965).
60. F. HUIJING, F. Q. NUTTALL, C. VILLAR-PALASI and J. LARNER, *Biochim. biophys. Acta* **177,** 204 (1969).
61. S. EL DEFRAWY and J. P. BUCKLEY, *J. Pharmac. exp. Ther.* **177,** 276 (1971).