presence of alkyl substituents on the phenolic ring do not alter deiodination.

4. Removal of the 4′-oxygen atom lowers deiodination of 3,5-di-iodothyronine analogues to less than 1%. Replacement by a 4′-amino group causes a return of activity, but replacement by a 4′-methyl group does not.

5. Substitution of a thio ether sulphur atom for the usual ether oxygen atom or the formation of the diphenyl analogues of 3,5-di-iodothyronine eliminate deiodination.

6. Ceric deiodination of 3,5-iodothyronines is probably initiated by oxidative removal of the outer phenolic ring.

The author is indebted to several individuals for supplying compounds included in this study: Dr H. A. Fevold (Travenol Division of Baxter Laboratories), Dr R. I. Meltzer (Warner–Lambert Research Institute), Dr R. Pitt-Rivers (National Institute for Medical Research), Dr E. C. Jorgensen (San Francisco Medical Center of University of California) and Dr W. F. J. Cuthbertson (Glaxo Laboratories Ltd.). Mr R. S. Wittner and Mr W. E. Crouch carried out much of the preliminary survey work and Dr G. S. Boyd aided with many valuable discussions. Thanks are due to Professor R. B. Fisher and the Biochemistry Department, University of Edinburgh, for hospitality during the tenure of a U.S. Public Health Service Special Fellowship. The work was supported by U.S. Public Health Service research grants A–1545 and A–3241.

REFERENCES

Barker, S. B. (1962). *Fed. Proc.* **21**, 635.
Block, R. J., Durrum, E. L. & Zweig, G. (1958). *Manual of Paper Chromatography and Paper Electrophoresis*, 2nd ed., p. 90. New York: Academic Press Inc.
Bowden, C. H., Maclagan, N. F. & Wilkinson, J. H. (1955). *Biochem. J.* **59**, 93.
Dubravčić, M. (1955). *Analyst*, **80**, 295.
Fletcher, K. & Stanley, P. G. (1956). *Nature, Lond.*, **175**, 730.
Galton, V. A. & Ingbar, S. H. (1962*a*). *Endocrinology*, **70**, 210.
Galton, V. A. & Ingbar, S. H. (1962*b*). *Endocrinology*, **70**, 622.
Lissitzky, S. (1960). *Bull. Soc. Chim. biol., Paris*, **42**, 1187.
Plaskett, L. G. (1961). *Biochem. J.* **78**, 652.
Tata, J. R. (1959). *Biochem. J.* **72**, 214.
Tata, J. R. (1960). *Biochem. J.* **77**, 214.
Tata, J. R. (1961). *Acta endocr., Copenhagen*, **37**, 125.

Biochem. J. (1964) **90**, 219

Nicotinamide Coenzyme Concentrations in Mammary Biopsy Samples from Ketotic Cows

BY D. S. KRONFELD AND FIORA RAGGI
Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, Kennett Square, Pa., U.S.A.

(*Received* 19 *July* 1963)

Tracer experiments in spontaneously ketotic cows have indicated defects in the reductive synthesis of milk fat from acetate and in the oxidation of glucose (Kronfeld, Kleiber & Lucas, 1959; Tombropoulos & Kleiber, 1961). Serum concentrations of metabolites were thought by Bach & Hibbitt (1959) to reflect interference with the citric acid cycle, notably with those reactions in which pyruvate and α-oxoglutarate are converted into citrate and succinate respectively, These impaired oxidative and reductive reactions all involve the nicotinamide–adenine nucleotide coenzymes. For this reason Kronfeld & Kleiber (1959) postulated that the simplest explanation for the concurrence of these metabolic defects in acetonaemia would be a shortage of all forms of these nicotinamide coenzymes. Alternatively, a primary defect in any one of these reactions could lead to defects in the others: for example, a primary defect in lipogenesis would be expected to lead to the accumulation of reduced forms of the coenzymes and a depletion of oxidized forms, and hence secondary impairment of the oxidative reactions in the pentose and citric acid cycles. A direct approach to possible causal relationships among these defects appeared to be the determination of nicotinamide–adenine nucleotide concentrations in the ketogenic tissue, in this case mammary gland (Kronfeld & Kleiber, 1959). A preliminary report has been presented (Kronfeld & Raggi, 1963).

METHODS

Coenzyme assay. Our fluorimetric assay follows that of Bassham, Birt, Hems & Loening (1959). It depends on the selective destruction of oxidized forms by alkali and of

reduced forms by acid, and on the specificity of alcohol dehydrogenase for NAD and of glucose 6-phosphate dehydrogenase for NADP. We repeated the results of Bassham *et al.* (1959) on rat-liver homogenates with ease, but experienced some difficulties with mammary homogenates, mainly loss of activity during the procedure and development of a yellow colour associated with high blanks. These were minimized by the following modifications: (i) blood and milk on the sample were washed off with cold buffer; (ii) nicotinamide (final concn. 20 mM) was added to the initial tris buffer; (iii) more dilute alkali (NaOH, final concn. 0·02 N) was used for the 'reduced' fraction, which was incubated at 60° instead of boiling; (iv) cysteine (final concn. 0·25 μM) was included in the alkaline incubation mixture; (v) both acidic (oxidized) and alkaline (reduced) fractions after incubation were centrifuged with hexane (1 ml./10 ml. of solution) at 26000*g* for 20 min. Fluorescence was measured in a Turner fluorimeter model 111, with primary filter 7–60 and secondary 75 (G. K. Turner Associates, Palo Alto, Calif., U.S.A.).

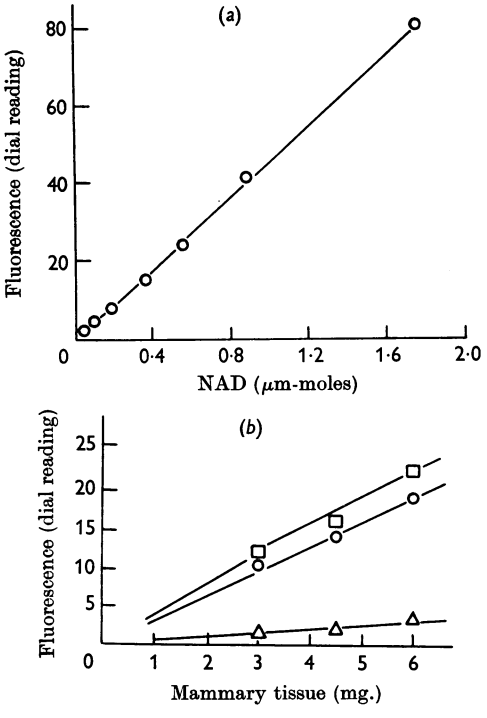

Fig. 1. Fluorescence developed by increasing amounts of (*a*) standardized NAD and (*b*) mammary homogenate: ○, NAD; △, $NADH_2$; □, NAD + $NADH_2$.

The method then gave linear results with standard NAD and various dilutions of mammary homogenate (Fig. 1). Recovery of added NAD was 90%, and that of NADP was just over 100% (Table 1). NAD (Sigma Chemical Co., St Louis, Mo., U.S.A.) was standardized spectrophotometrically with alcohol dehydrogenase (Boehringer und Soehne, Mannheim, Germany) according to the method of Racker (1950). NADP (Sigma) was also standardized spectrophotometrically, by using glucose 6-phosphate dehydrogenase (Sigma) according to the method of Glock & McLean (1953). The results of the assays were expressed as μm-moles of coenzyme/g. of protein. The protein was determined by a biuret method (similar to that used by Gornall, Bardawill & David, 1949) on the supernatant from the homogenate after centrifuging at 600*g* for 10 min. The standard deviation on duplicate assays of protein was less than 4% of the mean. Coenzyme assays on the biopsy samples were done in triplicate. Precision was calculated as the mean standard deviation within assays by analysis of variance, and was 4% of the mean for NAD, 13% for NADP (Table 2). The *F* test showed that variance between assays was highly significantly larger than variance within assays (Table 2).

Blood analysis. Jugular blood samples were collected by using fluoride–oxalate anticoagulant. They were centrifuged at 600*g* for 30 min., the packed-cell volume was read, and the plasma was tested semi-quantitatively for acetone plus acetoacetate (Dumm & Shipley, 1946). The plasma was then frozen until the subsequent determination of glucose (Campbell & Kronfeld, 1961) and of non-esterified fatty acids (Annison, 1960).

Cows. Six cows were purchased from farmers on a veterinarian's diagnosis of primary spontaneous uncomplicated acetonaemia. Each cow was transported to our Clinic by ambulance, and left for a few hours until quiet. A blood sample was taken and then the udder biopsy was performed. Medical treatment was commenced the following day, except with the cow K4, which was showing no clinical signs or ketonaemia (Table 3). Cow K5 was

Table 1. *Recovery of coenzymes*

A known amount of a coenzyme was added to a portion of mammary homogenate. The coenzyme was assayed by the method described in the text.

	Coenzyme added (μm-moles)	Homogenate (μm-moles)	Homogenate and coenzyme (μm-moles)	Coenzyme recovered (μm-moles)	Recovery (% of added)
NAD	56	61	111	50	89
	76	114	183	69	91
$NADH_2$	33	6	35	29	88
	63	11	76	65	103
NADP	60	6	66	60	100
	21	23	45	22	105
$NADPH_2$	52	6	55	49	94
	155	13	167	154	99

Table 2. *Precision of coenzyme assays*

The mean standard deviation of triplicate assays on 11 mammary homogenates was calculated by analysis of variance. Adequacy of precision was evaluated by F, the ratio of the estimated variance between assays to the variance within assays.

Coenzyme	Standard deviation within assays (μm-moles/g. of protein)	Standard deviation within assays (% of mean)	F	P
NAD	45·0	4·0	418	<0·001
$NADH_2$	30·7	7·1	146	<0·001
NADP	16·5	13·4	58	<0·001
$NADPH_2$	39·9	13·1	66	<0·001

exhibiting typical signs of acetonaemia at the time of her first biopsy. Five days later she developed a displaced abomasum, and remained chronically ketotic for 7 weeks. In cows K1, K2, K3 and K7, treatment with glucose, glucocorticoid or nicotinic acid was followed by remission of clinical signs and return of the concentrations of plasma glucose, ketone bodies and non-esterified fatty acids to the normal ranges. When this normal condition was established for 2 or more days, a second biopsy was performed for a control assay. A third biopsy was performed on cow K3, and an additional control assay was done on cow 27 which had not had acetonaemia. Although further control groups (normal cows that had never exhibited acetonaemia and normal cows before and after therapy) may have been desirable, these were not done because of the expense involved in purchasing cows and because much of the essential information sought appears to have emerged from the present experiments.

Mammary biopsy. The udder was milked out completely just before the operation. Anaesthesia was accomplished by paravertebral or epidural block (or both) with procaine. A vertical skin incision about 7 cm. long was made on the lateral aspect of a fore-quarter, and then a single cut made about 3 cm. deep through the fascia into glandular tissue. The edge of the fascia and glandular tissue was grasped with rat-tooth forceps, facilitating the rapid excision of a boat-shaped piece of tissue about 5 cm. × 2 cm. × 2 cm. It was washed immediately in cold buffer, and any adipose tissue was trimmed off, leaving about 5–10 g. of glandular tissue. The elapsed time was about 1 min. Then haemostasis and closure followed routine surgical procedures.

RESULTS

The results of coenzyme assays are listed in Table 3. Any therapy that brought about clinical recovery resulted in a marked increase in nicotinamide coenzyme concentrations. The main differences between control and acetonaemic cows are easier to see in Table 4: concentrations of all forms of the nicotinamide–adenine nucleotides are much lower than normal during acetonaemia. If 'atypical' cases are also considered, the difference between their total coenzyme concentration and that in normal animals remains highly significant, but individual coenzyme concentrations are not consistently lower. For example, in cow K4, the $NADH_2$ concentration was higher in the first assay (540 μm-moles/g. of protein) than in either of the subsequent assays (367 and 377 μm-moles/g. of protein) (Table 3). There is little difference between the ratios of oxidized to reduced forms of the coenzymes in normal and ketotic cows (Table 5). The stage of lactation did not appear to affect the coenzyme concentrations (Fig. 2).

DISCUSSION

The results clearly support the hypothesis that the oxidative and reductive defects in carbohydrate and fat metabolism that occur together during acetonaemia are associated with a shortage of the nicotinamide coenzymes which are involved in the impaired reactions. Nicotinamide is produced in the rumen (McElroy & Goss, 1941) and in ruminant tissues (Johnson, Wiese, Mitchell & Nevens, 1947), and it is often assumed that ruminants would not suffer from a shortage of this vitamin. However, the rate of nicotinamide production has not been estimated, and may not always match its outflow in the milk, which is about 850 mg./l. (Spector, 1956). Highly productive dairy cows are prone to acetonaemia, and this has usually been attributed to a drain of carbohydrate (see, for example, Shaw, 1956), but it could just as likely involve a drain of other essential substances, including vitamins. In a very limited number of cases, we have found that nicotinic acid or nicotinamide given intravenously or intracisternally has been followed by a decrease in the ketonaemia and ketonuria (with less striking effects on the hypoglycaemia and clinical signs). It thus appears possible that the low coenzyme concentrations may be due to failure of absorption of the vitamin from the digestive tract. In any case, the formation of the entire group of nicotinamide-derived coenzymes, rather than the conversion of oxidized into reduced forms or vice versa, emerges as a problem in the metabolic basis of bovine acetonaemia.

We have assumed that a low concentration of the coenzymes is a sign of a 'shortage', and that it would exert a regulating or rate-limiting effect on the reaction involved. The latter appears to be well established for the pentose phosphate pathway, which is regulated by the supply of NADP in rat mammary gland (McLean, 1960). On the other hand, the problem of rate-regulation of lipogenesis from acetate is fraught with controversy. This has probably been accentuated by the study of various preparations of cell fractions, which might not precisely reflect the responsiveness of the intact cell. For example, the fatty acid-synthesizing

Table 3. *Clinico-chemical status and nicotinamide coenzymes of dairy cows*

Details are given in the text.

Cow	Breed	Age (years)	Lactation (days)	Clinical condition and subsequent treatment	Plasma concentrations: Acetone + acetoacetate (mg./100 ml.)	Plasma concentrations: Non-esterified fatty acids (m-equiv./l.)	Plasma concentrations: Glucose (mg./100 ml.)	Mammary protein (mg./g. fresh wt.)	Concn. of nicotinamide–adenine nucleotide coenzymes in mammary gland (μm-moles/g. of protein): NAD	$NADH_2$	NADP	$NADPH_2$	Total
K1	Guernsey	9	23	Acetonaemia; dexamethazone, glucose, nicotinic acid	25	1·06	57	201	489	461	79	50	1078
			51	Normal	0	0·24	53	129	1031	340	156	606	2133
K2	Guernsey	4	10	Acetonaemia; glucose	45	0·42	47	126	648	230	76	222	1176
			13	Normal	0	0·12	64	97	2304	540	141	464	3449
K3	Holstein	11	21	Acetonaemia; glucose, dexamethazone, dexamethazone plus insulin	55	1·05	36	179	673	141	54	115	983
			29	Normal	0	0·55	67	113	1479	630	139	308	2556
			33	Normal	0	0·34	55	—	1416	831	110	317	2674
K4	Guernsey	8	12	Doubtful case, no treatment	20	0·83	61	122	1101	540	18	261	1920
			15	Still doubtful, nicotinic acid	0	0·44	48	110	1277	367	29	249	1922
			19	Abomasal displacement?	0	0·14	58	67	1670	377	73	273	2393
K5	Holstein	6	10	Acetonaemia; glucose	45	1·48	30	114	810	395	76	190	1471
			29	Chronic acetonaemia with displaced abomasum	5	0·14	61	147	778	184	147	338	1447
			52		5	0·16	52	236	647	380	52	180	1259
K7	Guernsey	9	12	Acetonaemia; nicotinic acid, glucose, dexamethazone	80	0·31	46	136	900	169	54	128	1251
			31	Normal	0	0·26	54	160	1319	418	141	549	2427
27	Holstein	7	253	Normal	0	0·18	63	167	1489	629	324	376	2818

Table 4. *Summary of nicotinamide coenzyme concentrations in mammary homogenates*

Only assays on five typical cases of acetonaemia (see Table 3) and six apparently normal cows are included. Concentrations are given as means ± S.E.M.

	Concn. (μm-moles/g. of protein)			
	Normal	Ketotic	*t*	*P*
NAD	1506 ± 174	704 ± 70	4·266	<0·005
$NADH_2$	565 ± 70	279 ± 62	3·065	<0·02
NADP	168 ± 31	68 ± 6	3·141	<0·02
$NADPH_2$	437 ± 50	141 ± 30	5·016	<0·001
Total	2676 ± 181	1192 ± 83	7·420	<0·001

Table 5. *Ratios of oxidized to reduced forms of nicotinamide coenzymes in mammary homogenates*

Only assays on five typical cases of acetonaemia (see Table 3) and six apparently normal cows are included (as in Table 4).

	Normal	Ketotic	*t*	*P*
Ratio of means (from Table 4)				
NAD:$NADH_2$	2·66	2·52	—	—
NADP:$NADPH_2$	0·38	0·48	—	—
Arithmetic mean ± S.E.M. of ratios (calculated from Table 3)				
NAD:$NADH_2$	2·81 ± 0·36	3·20 ± 0·80	0·441	<0·7
NADP:$NADPH_2$	0·42 ± 0·09	0·64 ± 0·22	0·926	<0·4
Geometric mean ± S.E.M. of ratios (calculated from Table 3)				
NAD:$NADH_2$	2·70 + 0·30, − 0·27	2·77 + 0·96, − 0·71	0·088	>0·9
NADP:$NADPH_2$	0·39 + 0·08, − 0·06	0·52 + 0·17, − 0·13	0·939	<0·4

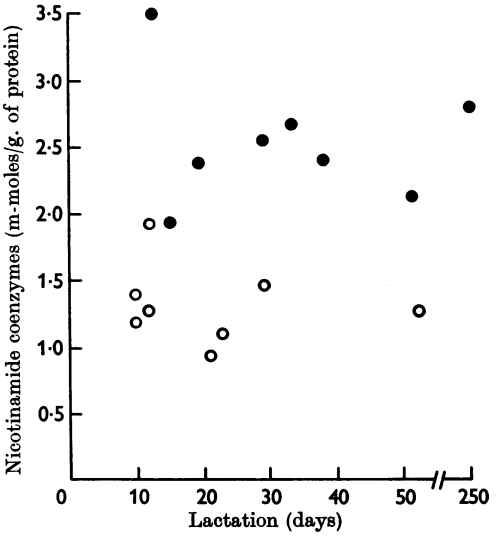

Fig. 2. Effect of lactation on total nicotinamide coenzyme concentration in bovine mammary homogenates: ●, normal cows; ○, ketotic cows.

system extracted from lactating-rat mammary gland by Abraham, Mathes & Chaikoff (1961) had an absolute requirement for citrate which could not be replaced by glucose 6-phosphate, but the ostensibly similar preparation of Dils & Popják (1962) was not stimulated by citrate in the presence of optimum concentrations of NADP, glucose 6-phosphate and $NADH_2$. The possibility that lipogenesis is regulated by the availability of $NADPH_2$ is suggested by its requirement for both the $\alpha\beta$-unsaturated-acyl-CoA-reductase and palmitate-synthetase systems, and by the parallel changes in lipogenic activity and pentose-cycle activity (which generates $NADPH_2$) in a number of situations: e.g. starvation, diabetes, acetonaemia and growth hormone treatment, which diminish their activity, and refeeding, some forms of obesity and insulin treatment, which enhance their activity. However, in liver from starved or diabetic rats, a deficiency of $NADPH_2$ now seems unlikely to be the major cause of defective lipogenesis because 'replacement' or provision of $NADPH_2$ does not fully correct the defect in subcellular preparations (Abrahams, Mathes & Chaikoff, 1960), and because the rate-limiting step appears to lie in acetyl-CoA carboxylase (Numa, Matsuhachi & Lynen, 1961), which does not require $NADPH_2$. In conflict again, the preparations of Gibson & Hubbard (1960) and of Wakil & Bressler (1962) appear to be defective in the palmitate-synthetase system. In diabetic or ketotic mammary gland, direct enzyme assays and cofactor-replacement experiments have yet to be done. But incorporation of [^{14}C]acetate into milk fatty acids in ketotic cows indicates that the defect lies between C_4 and C_{12} (Thin, Kleiber & Kronfeld, 1962), i.e. at a level at which $NADPH_2$ is required. And the present coenzyme assays show

a highly significant low concentration of $NADPH_2$ in mammary-gland homogenates from ketotic cows. These two items fit with the concept that a shortage of $NADPH_2$ might be a factor contributing to the lipogenic defect in ketotic bovine mammary gland. If this lipogenic defect were due entirely to some other cause, $NADPH_2$ might be expected to accumulate, and this was not found in the ketotic homogenates.

The possibility that a low NAD concentration might limit the rate of oxidative decarboxylation has not received as much attention. Bach & Hibbitt (1959), on finding abnormal serum concentrations of citric acid-cycle intermediaries in ketotic cows, suggested interference with oxidative decarboxylative reactions, and suspected deficiency of enzymic cofactors, such as coenzyme A and adenosine triphosphate. Since oxidative decarboxylation also requires NAD, and since we have found a low concentration of this coenzyme, its deficiency should also be suspected of contributing to the impairment of these oxidative reactions.

SUMMARY

1. A fluorimetric method for assay of nicotinamide–adenine nucleotide coenzymes has been developed for mammary tissue. Modifications of a method previously described for rat liver were devised to minimize both loss of activity and the development of a yellow colour. The results obtained were then linear, recovery of added coenzymes was between 88 and 105 %, and precision ranged from 4 % of the mean for NAD to 13 % for $NADPH_2$.

2. Six cows diagnosed as having spontaneous uncomplicated acetonaemia were purchased. Mammary biopsies were performed, yielding 5–10 g. of tissue, which was then assayed for the coenzymes. On the next day the cow was treated with glucose, dexamethazone or nicotinic acid, except for a doubtful case, which was not treated. When recovery of the cow appeared complete, one or two more biopsies and assays were performed to serve as controls.

3. Comparison of typical cases with apparently fully recovered cows showed that the concentrations of all forms of the nicotinamide–adenine nucleotide coenzymes was lower than normal during acetonaemia, although the ratio of oxidized to reduced forms was not changed. These results were interpreted to mean that a shortage of all forms of these coenzymes, rather than failure to convert oxidized into reduced forms or vice versa, is a metabolic difficulty in this disease. Such a shortage could provide a common factor for the impaired oxidative and reductive reactions which occur in bovine acetonaemia.

This work was supported by the U.S. Public Health Service Grant AM-04927 from the National Institutes of Health. We thank Professor R. E. Davies for helping us to modify the coenzyme assay, and veterinarians Dr H. L. Easterbrooks, Dr D. Haverstick, Dr J. W. McCahon and Dr R. H. Stoneback who helped us to purchase the ketotic cows.

REFERENCES

Abraham, S., Mathes, K. J. & Chaikoff, I. L. (1960). *J. biol. Chem.* **235**, 2551.
Abraham, S., Mathes, K. J. & Chaikoff, I. L. (1961). *Biochim. biophys. Acta*, **49**, 268.
Annison, E. F. (1960). *Aust. J. agric. Res.* **11**, 58.
Bach, S. J. & Hibbitt, K. G. (1959). *Biochem. J.* **72**, 87.
Bassham, J. A., Birt, L. M., Hems, R. & Loening, U. E. (1959). *Biochem. J.* **73**, 491.
Campbell, L. A. & Kronfeld, D. S. (1961). *Amer. J. vet. Res.* **22**, 587.
Dils, R. & Popják, G. (1962). *Biochem. J.* **83**, 41.
Dumm, R. M. & Shipley, R. A. (1946). *J. Lab. clin. Med.* **31**, 1162.
Gibson, D. M. & Hubbard, D. D. (1960). *Biochem. biophys. Res. Commun.* **3**, 531.
Glock, G. E. & McLean, P. (1953). *Biochem. J.* **55**, 400.
Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
Johnson, B. C., Wiese, A. C., Mitchell, H. H. & Nevens, W. B. (1947). *J. biol. Chem.* **167**, 729.
Kronfeld, D. S. & Kleiber, M. (1959). *J. appl. Physiol.* **14**, 1033.
Kronfeld, D. S., Kleiber, M. & Lucas, J. M. (1959). *J. appl. Physiol.* **14**, 1029.
Kronfeld, D. S. & Raggi, F. (1963). *Fed. Proc.* **22**, 471.
McElroy, L. W. & Goss, H. (1941). *J. Nutr.* **21**, 163.
McLean, P. (1960). *Biochim. biophys. Acta*, **37**, 296.
Numa, S., Matsuhachi, M. & Lynen, F. (1961). *Biochem. Z.* **334**, 203.
Racker, E. (1950). *J. biol. Chem.* **184**, 313.
Shaw, J. C. (1956). *J. Dairy Sci.* **39**, 402.
Spector, W. S. (1956). *Handbook of Biological Data*, p. 50. Philadelphia: W. B. Saunders Co.
Thin, C., Kleiber, M. & Kronfeld, D. S. (1962). *Amer. J. vet. Res.* **23**, 544.
Tombropoulos, E. G. & Kleiber, M. (1961). *Biochem. J.* **80**, 414.
Wakil, S. J. & Bressler, R. (1962). *J. biol. Chem.* **237**, 687.