PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **311th Meeting** *of the Biochemical Society was held in the Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E.* 1, *on Friday,* 24 *October* 1952, *when the following papers were read:*

COMMUNICATIONS

Malonate Inhibition of Succinic Dehydrogenase. By M. B. Thorn. (*Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E.* 1)

The mechanism of competitive inhibition of succinic dehydrogenase by malonate (Quastel & Wooldridge, 1928) may be represented:

$$E+S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E+\text{products}, \quad K_m=\frac{k_2+k_3}{k_1},$$

$$E+I \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EI, \quad K_i=\frac{k_5}{k_4},$$

where E is succinic dehydrogenase, ES the enzyme-succinate complex, and EI the enzyme-malonate complex. 'Relative affinity' means the ratio K_m/K_i, which is approximately equal to the ratio $[S]/[I]$ at 50 % inhibition of the catalysed reaction, provided that $[S]$ is much greater than K_m.

Conflicting values have been obtained for the relative affinity of succinic dehydrogenase for succinate and malonate. Krebs & Johnson (1948) obtained a figure of 10 with minced pigeon-breast muscle. Potter & DuBois (1943), using rat-liver homogenate, report a value of 50. Thorn (1951), using heart-muscle preparation with methylene blue and cyanide, obtained figures from 4·7 to 7·5 with methylene blue from 4×10^{-4} to 10^{-3} M. Krebs, Gurin & Eggleston (1952) found a value of 60 with yeast preparations.

The work to be reported was carried out using heart-muscle preparations (Keilin & Hartree, 1947). Succinic dehydrogenase activity was measured in two ways. The first method was by reduction of $K_3Fe(CN)_6$ in the presence of cyanide, using the Spekker absorptiometer (Ilford 601 filter) to measure the rate of decrease of optical density in the region of 400 mμ (essentially the method of Slater & Bonner, 1952). The second method was manometric, using methylene blue in the presence of cyanide. Succinic oxidase activity was measured manometrically.

K_m and K_i were determined (Lineweaver & Burk, 1934) using $K_3Fe(CN)_6$. K_m of succinic dehydrogenase in six preparations was between $2{\cdot}5 \times 10^{-4}$ and $5{\cdot}3 \times 10^{-4}$. K_i for malonate was between $5{\cdot}4 \times 10^{-6}$ and $9{\cdot}8 \times 10^{-6}$. Corresponding values gave calculated K_m/K_i ratios of about 50. In one preparation K_m/K_i, determined directly, was 58 for succinic oxidase, and 60 for succinic dehydrogenase by the $K_3Fe(CN)_6$ method. This agreed with a value of 57 calculated from separate determinations of K_m and K_i. When the ratio was determined for succinic dehydrogenase by the methylene-blue method, it varied from 6·5 to 24 with a 20-fold increase in methylene-blue concentration.

These results can be explained according to the theory worked out by Slater & Bonner (1952) for fluoride and phosphate. Values of the constants k_1, k_2 and k_3 were determined, and agreed substantially with those of Slater & Bonner. The results will be discussed.

The author gratefully acknowledges his indebtedness to Dr E. C. Slater and Dr W. D. Bonner for access to a draft of their paper before publication.

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The Adeninenucleotide Specificity of Oxidative Phosphorylation. By E. C. SLATER and F. A. HOLTON. (*Molteno Institute, University of Cambridge*)

Although recent studies have shown that adenosinediphosphate (ADP) is often phosphorylated by respiring tissue preparations much more rapidly than adenosinemonophosphate (AMP) (Slater, 1950; Barkulis & Lehninger, 1951; Kielley & Kielley, 1951; Lindberg & Ernster, 1952), they have not determined unequivocally the adeninenucleotide specificity of the oxidative phosphorylation reaction itself. In view of the rapid phosphorylation of AMP found in earlier work, the possibility remained that the oxidative phosphorylation system is not completely specific for ADP, but reacts slowly with AMP. Once ADP was formed by this reaction, it would react in preference to the AMP. This possibility has been tested by measuring the formation of energy-rich phosphate groups ($\sim$P) by a sensitive enzymic method (Slater, 1951), during the oxidation of α-ketoglutarate by a very dilute preparation (0·12 mg. protein/ml.) of cat heart-muscle sarcosomes (mitochondria). In contrast to liver mitochondria, cat-heart sarcosomes possess only very slight myokinase activity, in the presence of fluoride and AMP, two inhibitors of myokinase. The following results have been obtained:

(1) The relative rates of oxidation of α-ketoglutarate with various additions were: no addition, 46; +hexokinase+glucose, 75; +ADP, 100; +ADP+hexokinase+glucose, 100. The high rate of oxidation in the absence of ADP, especially with added hexokinase and glucose, is presumably due to the presence of endogenous $\sim$P (Green, Atchley, Nordmann & Tepley, 1949; Kielley & Kielley, 1951). With the enzyme concentrations used, the amount of endogenous $\sim$P was below the limits of detection by the analytical procedure. In the presence of hexokinase, the P:O ratio was 1·24 without added ADP and 2·72 with added ADP.

(2) When AMP was the only adenine nucleotide added, no $\sim$P ($<0{\cdot}02$ μmole) was detected, either in the absence or presence of hexokinase, even after 45 min., when 1·1 μmoles of hexosemonophosphate were formed in the presence of hexokinase. With ADP, the following values for $\sim$P were found after 1, 3 and 10 min.: 0·03, 0·08, 0·28; corresponding to a P:O ratio of 2·4 (cf. 3·0 with added hexokinase).

These results show that AMP is not directly phosphorylated in the oxidative phosphorylation reaction itself. Its rapid phosphorylation by respiring liver mitochondria must depend upon the formation of ATP from endogenous ADP, followed by the myokinase reaction (cf. Kielley & Kielley, 1951). Our conclusions are in opposition to those of Lindberg & Ernster (1952).

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Transfructosidation by Mould Invertase Preparations. By J. EDELMAN (*Research Institute of Plant Physiology, Imperial College, London, S.W.* 7) and F. J. BEALING (*Department of Biochemistry, University of Sheffield*)

Enzymes hitherto regarded as invertases appear to act primarily as transglycosidases (Blanchard & Albon, 1951; Bacon & Edelman, 1951*a*; Edelman & Bacon, 1951; Bealing & Bacon, 1951; Fischer, Kohtès & Fellig, 1951; Bacon, 1952). The use of radioactive glucose has further elucidated the reactions involved.

Samples (3 μl.) of a digest incubated at 20° and containing 10 mg. radioactive glucose, 15 mg. inactive sucrose, 0·12 ml. 0·011 M-acetate buffer pH 5·0, and 0·08 ml. mould invertase preparation (from *Penicillium spinulosum*, see Bealing & Bacon, 1952) were chromatographed on paper sheets (Partridge, 1948). Using a thin end-window Geiger-Müller counter directly on the paper, the counts/min. (c/m) of the glucose spots were: after 3 min. incubation 4133, 30 min. 3350, 60 min. 3439, 120 min. 3624. The corresponding c/m of the sucrose spots were 218, 551, 546 and 340, and of spot α (?trisaccharide consisting of two fructose and one glucose units, see Bealing & Bacon, 1952) were 8, 91, 212 and 377. Spot β (?tetrasaccharide consisting of three fructose and one glucose units) was present in the 60 and 120 min. samples, the c/m being 10 and 62 respectively. Recoveries of the c/m applied were 95–97 %. At 120 min. these values represented 16·8 (sucrose),

11·4 (trisaccharide) and 8·8 (tetrasaccharide) c/m/μg. combined glucose, assuming each spot to contain only the postulated oligosaccharide. Two-dimensional chromatography with hydrolysis of oligosaccharides after the first development (Bacon & Edelman, 1951*b*) showed that only the glucose parts of the molecules were labelled.

Isolation of glucose-labelled sucrose from a similar digest was accomplished by adding 25 mg. carrier sucrose after 30 min. and separating the sugars on a charcoal-Celite column (Whistler & Durso, 1950); crystals obtained were identified as sucrose from X-ray powder photographs. The c/m/μg. after crystallization and recrystallization were 3·48 and 3·64 respectively.

These results support the hypothesis that mould invertase transfers fructose residues to suitable acceptors, including water, glucose and sucrose (Bealing & Bacon, 1951); transfer to glucose gives sucrose, and further transfer to glucose-labelled sucrose gives similarly labelled higher saccharides (spots α, β). Thus addition of glucose to an enzyme-sucrose digest decreases the rate of sucrose breakdown owing to the increased rate of reformation of the initial substrate; this would explain the long-known inhibition of mould (taka-) invertase by glucose (see Neuberg & Mandl, 1950).

We thank Dr H. K. Porter for the gift of radioactive glucose, and Dr A. J. E. Welch and the Royal Society for the X-ray photographs of sucrose.

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The Conversion of Naphthalene to 1:2-Dihydronaphthalene-1:2-diol monoglucuronide in the Rabbit. By E. D. S. Corner, F. S. Billett and L. Young. (*Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E.* 1)

From the urine of rats and rabbits dosed with anthracene, Boyland & Levi (1935, 1936) isolated 1:2-dihydroanthracene-1:2-diol and also the monoglucuronide of this compound. 1:2-Dihydronaphthalene-1:2-diol is excreted in the urine of rats (Young, 1947) and rabbits (Booth & Boyland, 1949) following the administration of naphthalene, but the excretion of a conjugated form of this diol has not hitherto been reported.

In the present work it was found that although there was little increase in the ethereal sulphate content of the urine of rabbits after they had been dosed with naphthalene, there was a marked increase in the glucuronide content of the urine. This led to an investigation of the possibility that the urine of the dosed animals contained a glucuronide of 1:2-dihydronaphthalene-1:2-diol. From the urine of these animals it was found possible to obtain a powder which contained glucuronide. Attempts to separate a pure glucuronide from this powder always resulted in the formation of gums which could not be induced to crystallize. When the powder was treated with ethereal diazomethane followed by acetylation of the product, however, a crystalline compound was obtained, the analysis of which corresponded to that of the tetra-acetyl derivative of 1:2-dihydronaphthalene-1:2-diol monoglucuronide methyl ester; m.p. 209°; $[\alpha]_D^{20°} = +94°$ (*c*, 1% in chloroform). Found: C, 57·6; H, 5·6. $C_{25}H_{28}O_{12}$ requires C, 57·7; H, 5·5%.

Whereas the powder prepared from the urine contained no free 1:2-dihydronaphthalene-1:2-diol, this compound was present after treatment of the powder with β-glucuronidase. The diol thus liberated had the following properties: m.p. 127°; $[\alpha]_D^{25°} = +158°$ (*c*, 1% in ethanol), and it was identical with the dextrorotatory diol obtained by Booth & Boyland (1949) from the urine of rabbits dosed with naphthalene.

The evidence obtained suggests, therefore, that rabbits excrete a monoglucuronide of 1:2-dihydronaphthalene-1:2-diol after they have been dosed with naphthalene. The glucuronic acid of the conjugated compound appears to be attached to the 1-position of the naphthalene ring structure, for acid treatment of the glucuronide-containing powder resulted in the formation of 1-naphthylglucuronide.

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Stereochemical Aspects of the Metabolism of Ethylbenzene. By J. N. SMITH, R. H. SMITHIES and R. T. WILLIAMS. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W.* 2)

Ethylbenzene is hydroxylated in the body at the β-carbon of the side chain (Neubauer, 1901; Thierfelder & Daiber, 1923) to form phenylmethylcarbinol. Acetophenone is reduced *in vivo* to phenylmethylcarbinol which is excreted as a glucuronide (Thierfelder & Daiber, 1923). A possible mechanism for this hydroxylation is

$$PhCH_2CH_3 \rightarrow PhCH{:}CH_2 \rightarrow PhCOCH_3 \rightarrow PhCHOHCH_3.$$

The glucuronic acid conjugations of equivalent doses of the above compounds in rabbits were measured and found to be: ethylbenzene, 32; styrene, 16; acetophenone, 47; (±)-phenylmethylcarbinol, 50% of the dose. The conjugation of β-phenylethanol, $PhCH_2CH_2OH$, was low (7%), the main metabolites being phenylacetic acid conjugates.

The low value for the conjugation of styrene suggests that it is not a major intermediate in ethylbenzene hydroxylation.

Acetophenone also seems to be ruled out as a major intermediate because the glucuronide isolated from the urine of rabbits fed with acetophenone was entirely that of (−)-phenylmethylcarbinol (isolated as *triacetyl* β-(−)-*phenylmethylcarbinyl*-D-*glucuronide methyl ester*, m.p. 113° and $[\alpha]_D^{20°}$ −83° in $CHCl_3$). The reduction of acetophenone was thus asymmetric. The triacetyl methyl ester from ethylbenzene urine had $[\alpha]$ −50·2°, from which the (−)diastereoisomer (m.p. 113° and $[\alpha]_D$ −83°) was isolated by fractional crystallization and an impure (+)diastereoisomer (m.p. 130–136° and $[\alpha]_D$ −10°). The hydroxylation of ethylbenzene *in vivo* thus produced both isomers of phenylmethylcarbinol.

On feeding (±)-phenylmethylcarbinol, the isolated triacetyl methyl ester of the glucuronide had $[\alpha]_D$ −50°. Fractional crystallization yielded the pure (−)isomer ($[\alpha]_D$ −83·5°) in good yield and a small amount of impure (+)isomer ($[\alpha]_D$ −17·8°).

A possible mechanism of hydroxylation is abstraction of a hydrogen atom from the β-carbon by an enzyme and then addition of ·OH to the resulting free radical thus:

$$PhCH_2CH_3 \xrightarrow{-H\cdot} Ph\dot{C}HCH_3 \xrightarrow{+\cdot OH} (\pm)PhCHOHCH_3.$$

The oxidation of ethylbenzene to phenylmethylcarbinol and acetophenone by free radicals has been shown to occur in purely chemical systems (Emerson *et al.* 1948; Merz & Waters, 1949).

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Glucosulphatase Activity in Marine Molluscs. By K. S. DODGSON and B. SPENCER. (*Physiology Institute, Newport Road, Cardiff*)

The existence in certain tropical marine molluscs of a sulphatase capable of liberating sulphuric acid from glucose-6-monosulphate has been reported by Soda (1936), who suggested the name glucosulphatase for the enzyme. It has not been established with certainty that molluscs common to British waters possess this enzyme, although Percival (1949) claims some indications of its presence in these organisms.

A number of herbivorous and carnivorous molluscs have been examined in this laboratory, but only the large periwinkle (*Littorina littorea*) possesses appreciable glucosulphatase activity. A crude enzyme concentrate has been obtained by preparing an acetone-dried powder of periwinkle viscera and subsequent fractionation of an aqueous homogenate of this powder with acetone. The final product shows considerable glucosulphatase activity as determined by the method of Dodgson & Spencer (1952); 1 g. of the powder liberating 48·8 mg. SO_4 from approximately 0·03 M-potassium glucose-6-sulphate in 0·5 M-acetate buffer, pH 5·8, in 1 hr. at 37·5°. The concentrate also possesses appreciable β-glucuronidase and aryl- and chondro-sulphatase activity.

Maximum enzyme activity towards potassium

glucose-6-sulphate occurs at pH 5·8 when the substrate concentration is approximately 0·01 M. Preliminary experiments indicate the optimum substrate concentration to be greater than 0·01 M, but this point has not been pursued further, since there are indications that the substrate is not homogeneous. When prepared by the method of Duff (1949), barium and potassium glucose-6-sulphates are hydrolysed by 0·1 M-hydrazine at pH 5·5 and at 37·5° to an extent of about 15 % in 36 hr., the degree of hydrolysis varying slightly from preparation to preparation. No further hydrolysis can be achieved either by prolonged incubation or by addition of more hydrazine. On the other hand, addition of more substrate results in a further hydrolysis of about 15 % of the extra material. These findings agree with those of Egami (1938) for barium glucose-6-sulphate prepared by the method of Soda (1933). Soda & Egami (1942) noted that this partial hydrolysis could be almost completely eliminated by preparing glucose-6-sulphate via a fractionally crystallized brucine salt, and concluded that sulphation of glucose by the normal procedures gave more than one monosulphated product.

The preparation of pure potassium glucose-6-sulphate by a more convenient method is being studied in this laboratory before continuing enzyme studies with this substrate.

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Composition and Cytology of *Nitrosomonas*. By T. Hofman and H. Lees. (*Department of Biological Chemistry, University of Aberdeen*)

Pure cultures of *Nitrosomonas* were grown on a medium consisting chiefly of calcium and magnesium carbonates, phosphate and ammonium sulphate (Lees, 1952).

For amino-acid and sugar analyses the insoluble inorganic salts were dissolved in 5 % acetic acid and the washed organisms were hydrolysed with 6N-hydrochloric acid and with sulphuric acid respectively. The hydrochloric acid hydrolysate was analysed for total nitrogen, amino nitrogen (Pope & Stevens, 1939) and free ammonia. Two-dimensional paper chromatograms (phenol/butanol-acetic acid) were run for the identification of the amino-acids present. The separation of the leucine isomers was achieved by running a one-dimensional paper chromatogram in *tert.*-amyl alcohol in the presence of diethylamine (Work, 1949). The method of Fowden (1951) with small modifications was used for the determination of the amino-acids. The following results in terms of amino nitrogen as percentage of total nitrogen were obtained: aspartic acid 5, glutamic acid 8, serine 2, glycine 8, threonine 4, alanine 8, tyrosine 1, leucine 3, isoleucine 4, valine 7, arginine 2, histidine 1, lysine 4, phenylalanine 4, methionine 2, proline 4, ammonia 8.

Cystine was present in small amounts but was not estimated.

The amino-acid pattern is very similar to that obtained from a hydrolysate of *C. diphtheriae* (Work, 1949) except that no diaminopimelic acid has been found. No unidentified spots were detected on the chromatograms.

One- and two-dimensional chromatograms of a sulphuric acid hydrolysate revealed the presence of four sugars which were chromatographically identical with galactose, ribose, rhamnose and xylose, the latter being present only in traces. No glucose was found. Chromatograms sprayed with *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950) revealed an additional spot with a strong bluish fluorescence in the ultraviolet light. Its identity has not so far been established.

Electronmicrographs—taken by Mr J. A. Gard, ChemistryDepartment—showed that the organisms which were freed of the insoluble inorganic salts by fractional centrifugation possess one 'nuclear body' in each cell. Some photographs gave evidence of further organization inside this body. The formation of zooglea was clearly observed.

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The Chromatography of Bile Pigments with Particular Reference to the Van den Bergh Reaction. By P. G. COLE and G. H. LATHE. (*Bernhard Baron Memorial Research Laboratories, Queen Charlotte's Maternity Hospital, London, W.* 6)

Previous workers have noted the tenacity with which adsorption columns retain bile pigments unless they have first been esterified. To avoid this, in view of the instability of bile pigments in the mildest chemical procedures, separation of bile pigments has been attempted on partition columns.

For a preliminary separation of pigments a reverse phase column of silicone-treated Kieselguhr (Howard & Martin, 1950), using the two phases from 25 % CCl_4, 25 % $CHCl_3$, 38 % MeOH, 6 % water and 6 % (v/v/v/v) pH 6 phosphate buffer (Cole, 1933) is satisfactory. With a ratio of mobile phase to stationary phase of 6, bilirubin moves as a discrete band with an R_F of 0·6. Under the same conditions human autopsy bile yields a group of pigments, most of which move more rapidly.

Pigments were extracted from jaundice sera by the addition of 0·18 vol. of saturated ammonium sulphate and 2·5 vol. of ethanol, followed by centrifuging. Extracts from sera of patients with obstructive jaundice showed two yellow bands, a prominent one faster than bilirubin, and a small one moving as slow as, or slower than, bilirubin. Extracts of sera from cases of haemolytic jaundice (of the newborn) showed similar bands, the slower being much more pronounced, and the polar one being greatly reduced, as compared with sera from cases of obstructive jaundice.

The polar band, moving faster than bilirubin, gives a direct van den Bergh reaction, while bilirubin (after passage through a column), and the slow-moving band from jaundice sera, react indirectly in the van den Bergh test.

The movement of the polar, direct-reacting band from sera from obstructive jaundice patients is too rapid on the above column for good resolution. When it is run on a reverse phase column of 50 % $CHCl_3$, 30 % MeOH, and 20 % H_2O (v/v/v), with a ratio of mobile phase to stationary phase of 3, the band splits into two pigments, both of which produce colour in the van den Bergh reaction. This has been confirmed on other columns, but an entirely satisfactory system has not yet been achieved.

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The Application of Counter-Current Methods to the Fractionation of Lipid Material from Brain. By P. G. COLE, G. H. LATHE and C. R. J. RUTHVEN. (*The Bernhard Baron Memorial Research Laboratories, Queen Charlotte's Maternity Hospital, London, W.* 6)

A solvent system (A) composed of CCl_4 62 %, MeOH 35 % and H_2O 3·15 % (v/v/v) was devised to minimize emulsion formation in the counter-current distribution of lipids. With this solvent system crude preparations of brain lecithin, cephalin, sphingomyelin and cerebroside, as well as crude brain lipid, were distributed through 49 plates (tubes) in the glass and polythene apparatus previously described (Lathe & Ruthven, 1951). In this apparatus the lower layer (non-polar in solvent A) was moved.

A comparison of the weight curves of distributions indicated that the cephalin fraction contained more polar material than the sphingomyelin and cerebroside, while lecithin showed peaks of both polar and intermediate polarity. The distribution of crude lipid from brain in system A gave rise to three groups of fractions, very polar (tubes 0–4), polar (tubes 5–17) and one of intermediate polarity (tubes 18–40).

For further separation of polar fractions, the polarity of solvent A was altered by the addition of chloroform and methylene chloride or alternatively the phase ratio (upper/lower) was changed from 1/1 to 1/4. For the fractions of intermediate polarity redistribution in a system modified from A by the addition of light petroleum led to separation of cholesterol from phospholipins.

The analysis of fractions for nitrogen, phosphorus, sugars, cholesterol, and the identification of bases by paper chromatography, showed that there was considerable overlapping of many components. Amino-acids, reducing material, and breakdown products were present in the most polar tubes (0–4) of the distribution of crude brain in system A. A lecithin fraction was probably included in these tubes. Phosphatidyl ethanolamine and phosphatidyl serine were spread through tubes 4–16. In the central region peaks were found as follows: sphingomyelin tube 21, lecithin tube 27, cerebroside tube 29 and cholesterol tube 35. A small amount of non-polar material was present in the position of neutral fat at tube 45.

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A New Brief Method of Estimating Net Protein Value. By A. E. BENDER and D. S. MILLER. (*The Crookes Laboratories, Ltd., Park Royal, London, N.W.* 10)

The conventional Thomas-Mitchell method of estimating the biological value (B.V.) of proteins is to measure the proportion of the absorbed N retained in the body. B.V. × digestibility gives the net protein value (N.P.V.).

Let N intake on test diet $= I_f$; N intake on non-protein (or 4·5 % egg protein) diet $= I_K$; total faecal N $= F$; metabolic faecal N $= M$; urinary N $= U$; endogenous urinary N $= E$; then, by definition,

$$\text{N.P.V.} = \frac{I_f-(F-M)-(U-E)}{I_f-(F-M)} \times \frac{I_f-(F-M)}{I_f}$$
$$= \frac{I_f-F+M-U+E}{I_f}.$$

Subtract I_K/I_f from both sides of the equation,

$$\text{N.P.V.} - \frac{I_K}{I_f} = \frac{(I_f-F-U)-(I_K-M-E)}{I_f}.$$

I_f-F-U is the gain in N by the animal fed a protein diet = final carcass N − initial carcass N $= B_f-B$. I_K-M-E is the change in N incurred by feeding non-protein diet (or 4·5 % egg protein) $= B_K-B$

$$\text{N.P.V.} - \frac{I_K}{I_f} = \frac{(B_f-B)-(B_K-B)}{I_f},$$
$$\text{N.P.V.} = \frac{B_f-B_K+I_K}{I_f}.$$

This implies the impossible requirement of a carcass N analysis on the same rat fed the non-protein and test diets. The carcass N on the non-protein diet may be estimated by the use of control animals which should be litter mates of equal weight to the test animals.

In practice four litters of eight rats are divided by litters into groups of four so that each group totals the same weight. One group is fed non-protein diet (or 4·5 % egg protein) and the remaining seven groups fed seven test proteins at the 10 % level for 10 days. The animals are killed and the body N determined by Kjeldahl digestion of the whole carcass. The analyses of the four rats in each group are combined and substituted in the equation above.

Seven estimations of casein made in three separate experiments gave 59, 60, 55, 56, 58, 60 and 57, i.e. 57·9 ± 0·7 (S.E.). Other results were: commercial egg albumen 82, whey proteins 86, commercial dog-fish meal 51, and extracted bran protein 57.

The values for casein were compared with that obtained by the slope method of Allison & Anderson (1945) feeding at 4, 8, 10, 12 % levels. This method gave a net protein value of 59. The rats fed at the 10 % level were used for the carcass analysis method and gave a value of 54.

Egg albumen and casein were evaluated by the full Thomas-Mitchell method according to the procedure of Henry, Kon & Watson (1937) which showed B.V. of albumen 81·8 ± 2·62, digestibility 97·9 ± 0·5 %, casein B.V. 66·5 ± 1·76, digestibility 98·9 ± 0·3 %. The carcass analysis assay carried out simultaneously gave N.P.V. 82 and 57 ± 1·5 (triplicate assay) respectively.

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Constancy of the N/H_2O Ratio of the Rat and its Use in the Determination of the Net Protein Value. By A. E. BENDER and D. S. MILLER. (*The Crookes Laboratories, Ltd., Park Royal, London, N.W.* 10)

The Thomas-Mitchell method (Mitchell, 1923–4) of estimating biological value involves large numbers of N analyses of both urine and faeces. The development described in the previous paper reduces this to a single analysis on the whole body of the animal. It is shown here that the estimation can be replaced by a determination of the water content of the animal.

It was shown by Moulton (1923) that body constituents, including the N and water content, are a constant proportion of the body when measured on a fat-free basis. It follows that the ratio of N/H_2O is a constant and therefore the N content of the body can be derived from the water content.

One hundred and forty-seven rats aged 33–57 days were analysed for total body N and water, and the correlation between this N/H_2O ratio and age was found to be expressed by $y = 2{\cdot}92 + 0{\cdot}024x$, where y is N/H_2O % and x the age in days. The correlation coefficient was 0·42 ($P < 0{\cdot}001$), indicating a substantially linear relation over this short age range. These rats had been fed a wide range of diets varying from non-protein for 10 days to 20 %

protein for 3 weeks without affecting the N/H_2O ratio. The ratio for a 38-day-old rat is 3·8, which compares with a value of 3·92–3·97 calculated from the data of Light, Smith, Smith & Anderson (1934) for six rats. We do not yet know whether this ratio measured on our black and white hooded colony is applicable to other colonies.

The measurement of the net protein value is carried out as described in the previous paper but with the body N analyses replaced by a dry weight determination. After the 10-day feeding period the animals are killed with chloroform and weighed. Incisions are made into the skull and thoracic and body cavities and the carcasses dried to constant weight at 105° (24 hr.). The water content is converted to N by multiplying by the appropriate ratio, taking account of the age of the animal. The body N figures are substituted in the equation,

$$\text{net protein value} = \frac{B_f - B_K + I_K}{I_f}.$$

The following net protein values were obtained by this method, the values in brackets being those obtained by an actual N analysis of the rats. Extracted bran protein 58 (57), whey proteins 89 (86), commercial dogfish meal 55 (48), mixture FW 73 (72), casein determined seven times $55·1 \pm 1·7$ ($57·9 \pm 0·7$), diet M determined three times $58·6 \pm 2·3$ ($56·6 \pm 2·2$), egg albumen 83 (82).

This method would permit estimation of body N on the same subject both before and after feeding test diets, i.e. N balances, without sacrificing the animal, simply by measuring the body water by one of the accepted methods (isotope dilution, urea or antipyrine) and might be applicable to man.

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The Incorporation of Labelled Phosphate into the Lipids of a Guinea-pig Brain Homogenate.

By R. M. C. Dawson (Betty Brookes Research Fellow). (*Department of Biochemistry, University of Oxford*)

Cell-free homogenates of guinea-pig brain have been incubated in Ringer solutions containing labelled phosphate (^{32}P) and measurements made of the uptake of radioactivity by the lipids. A simple technique was evolved for the extraction and purification of the phospholipins. A chloroform/methanol extract of these was equilibrated with 2 vol. of N-HCL or 0·25 M-$MgCl_2$ whereby protein matter and contaminating acid-soluble P passed into the aqueous phase. By this means the zero-time uptake of labelled phosphate was reduced to a few per cent of the full enzymic incorporation.

For maximum uptake of ^{32}P the system required the presence of Mg^{++}, adenylic acid, cytochrome *c*, oxidizable substrate and O_2. The optimum pH was near 6·5. Nearly all the uptake occurred in the first hour of an incubation at 37°, the subsequent incorporation being very slow. Anaerobic glycolysis supported the uptake of phosphate only to a limited extent. The presence of F^- greatly activated the incorporation when the homogenate was respiring with a substrate of pyruvate plus fumarate. The uptake of phosphate could be uncoupled from the respiration of the homogenate by the presence of 2:4-dinitrophenol, azide, methylene blue, arsenate, gramicidin, SO_4^-, Ca^{++}, and with hypertonic Ringer. The incorporation was also inhibited by glucose, creatine, inorganic P, and in some conditions by adenosinetriphosphate. The addition of a number of coenzymes and possible elementary precursors of the phospholipins had no effect on the uptake of labelled phosphate. These results suggest that the uptake of phosphate into the phospholipin fraction of brain homogenates is dependent upon oxidative phosphorylation: this being rate limiting for the incorporation.

In further experiments the brain phospholipins have been fractionated using alcoholic precipitation, MgO adsorption, and saponification. The results show that the uptake is localized in the 'kephalin' fraction with practically no synthesis of labelled lecithins or sphingomyelins. The uptake into phosphatidyl ethanolamine has been measured by a new method based on catalytic hydrolysis with $HgCl_2$, and the results confirm earlier ones which showed that the synthesis was negligible in brain homogenates (Norman & Dawson, 1952). This indicates that the incorporation is confined solely to a fraction containing phosphatidyl serine, diphosphoinositide and possibly phosphatidic acids.

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Two Selective Inhibitors of Cholinesterase. By L. AUSTIN and W. K. BERRY. (*Chemical Defence Experimental Establishment, Porton, Wilts*)

Two new inhibitors have been described which, in view of their reported high selectivity towards 'true' and 'pseudo' cholinesterases, appeared to be useful tools for examination of some ChE's which do not fit the conventional classification (Ord & Thompson, 1951; Earl & Thompson, 1952; Davies, Risley & Rutland, 1952). They are, bis(bis-*iso*propylamino)-phosphonous anhydride—*iso*OMPA—reported by Aldridge (1952) to be 10000 times more active against the 'pseudo' ChE of horse serum than against the 'true' ChE of horse erythrocytes: and 1–5-bis(4-allyl dimethylammonium phenyl)pentan-3-one dibromide—284C51—found by Fulton (1952) to inhibit 'true' ChE but not 'pseudo'.

Using the enzymes of human blood, we have found that *iso*OMPA irreversibly inactivates the plasma 'pseudo' ChE. It attacks the active centre, and the kinetics of inhibition are bimolecular. 284C51 is a competitive reversible inhibitor of the erythrocyte 'true' ChE.

The selectivity of the inhibitors (expressed as the ratio I_{50} cell ChE:I_{50} plasma ChE) has been measured using the blood of four mammalian species as sources of 'true' (erythrocyte) and 'pseudo' (plasma) ChE. Substrate studies indicated the absence of 'pseudo' ChE from the erythrocytes, and vice versa. The selectivity of each inhibitor was generally high, but species variations exist. It appears possible that appropriate concentrations of both inhibitors should inhibit one type of ChE 95% while affecting the other by not more than 5%.

The finding by Earl & Thompson (1952) that fowl plasma contains one ChE, as sensitive to inhibitors as 'pseudo' ChE but capable also of hydrolysing acetyl-β-methyl choline, has been confirmed.

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