[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the authors.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **322nd Meeting** *of the Biochemical Society was held in the Department of Chemical Pathology, Westminster Hospital Medical School, Horseferry Road, London, S.W.* 1, *on Saturday,* 17 *October* 1953, *at* 11 *a.m., when the following papers were read:*

COMMUNICATIONS

Phenylalanine and Tyrosine Metabolism in Patients with Phenylketonuria.

By R. J. Boscott and H. Bickel.* (*Anatomy Department, The Medical School, Birmingham and The Children's Hospital, Birmingham*)

The urine of patients with phenylketonuria is known to contain an excess of phenylalanine, phenyl pyruvic, phenyl acetic and phenyl lactic acids. Jervis (1953) adduces further evidence that the metabolic error lies in the inability of the liver to convert phenylalanine into tyrosine by *p*-hydroxylation.

We have studied chromatographically (Boscott, 1952*a*, *b*, *c*) the phenolic fractions I, II and III (Boscott, 1952*a*) of the hydrolysed and non-hydrolysed urines from two children with phenylketonuria maintained on (1) a normal diet, (2) a normal diet with supplements of DL-phenylalanine, and (3) a diet free from phenylalanine (described by Bickel, Gerrard & Hickmans, 1953). The urines of twelve more phenylketonuric patients on normal diets were also investigated. All of these subjects excreted in the urine *p*-hydroxyphenyl lactic acid and *p*-hydroxyphenyl acetic acid, both of which are present as abnormal tyrosine metabolites in the urines of scorbutic guinea pigs (Aterman, Boscott & Cooke, 1953). The methods employed do not allow for the detection of the labile *p*-hydroxyphenyl pyruvic acid which also may be present.

Chromatograms of fraction I further revealed the consistent presence of greatly abnormal amounts of compound 30, the chromatographic behaviour and colour reactions of which are identical with those of *o*-hydroxyphenyl acetic acid. The unidentified compound 28, which was always present in normal urines, was not found in the urines of phenylketonurics. Phenyl pyruvic acid was detected on the chromatograms of fraction I as a lemon-yellow azo-derivative, with a bluish halo, by spraying the paper with diazotized ICI 5091 in Na_2CO_3 solution, followed either by heating the paper or by again spraying with 2N aqueous KOH solution.

Fractions II and III consistently revealed large amounts of unidentified fluorescent phenolic compounds, not previously detected in the urines of 140 normal and hospitalized individuals. The urinary excretion of these compounds as well as of *o*-hydroxyphenyl acetic acid and phenyl pyruvic acid was increased after supplements of DL-phenylalanine were added to the diet, but greatly reduced or abolished when the diet contained no phenylalanine. The excretion of *p*-hydroxyphenyl acetic acid and *p*-hydroxyphenyl lactic acid remained virtually unchanged. The excretion of pyrocatechol in fraction II appeared to be less than that normally encountered.

Thus the metabolic error in phenylketonurics would appear to be more complex than has hitherto been recognized. The findings suggest that neither the further hydroxylation of *p*-hydroxyphenyl pyruvic acid nor of *o*-hydroxyphenyl pyruvic acid, to give 2:5-dihydroxyphenyl pyruvic acid with ultimate breakdown of the benzene ring, is possible in phenylketonuric patients. This concept would lend support to the theory of Penrose & Quastel (1937) in addition to that postulated by Jervis (1947, 1950).

It is suggested that *o*-hydroxyphenyl acetic acid and its probable precursors, *o*-hydroxyphenylalanine, *o*-hydroxyphenyl pyruvic acid and *o*-hydroxyphenylethylamine, should be examined as possible causal agents of the amentia present in phenylketonurics.

* In receipt of a grant from the Medical Research Council.

REFERENCES

Aterman, K., Boscott, R. J. & Cooke, W. T. (1953). *Biochem. J.* **55**, xvii.
Bickel, H., Gerrard, J. & Hickmans, E. M. (1953). *Lancet* (in the Press).
Boscott, R. J. (1952*a*). *2nd Int. Congr. Biochem. Abstr.* p. 349.
Boscott, R. J. (1952*b*). *Biochem. J.* **51**, xlv.
Boscott, R. J. (1952*c*). *Chem. & Ind.* p. 472.
Dann, M., Marples, E. & Levine, S. Z. (1943). *J. clin. Invest.* **22**, 87.
Jervis, G. A. (1947). *J. biol. Chem.* **169**, 651.
Jervis, G. A. (1950). *Proc. Soc. exp. Biol., N.Y.*, **75**, 83.
Jervis, G. A. (1953). *Proc. Soc. exp. Biol., N.Y.*, **82**, 514.
Penrose, L. S. & Quastel, J. H. (1937). *Biochem. J.* **31**, 226.

Alkyl Sulphides Evolved from Dog's Urine by Sodium Hydroxide. By F. CHALLENGER, D. LEAVER and MARGARET I. WHITAKER. (*Organic Chemistry Department, University of Leeds*)

Abel (1895) concluded on slender evidence that the compound evolved from dog's urine by boiling alkali was diethyl sulphide. Neuberg & Grosser (1905–6) claimed, without details, to have obtained the phosphotungstate and bismutho-iodide of $[Me.\overset{+}{S}.Et_2]\ \overset{-}{O}H$ from the urine. M. I. Whitaker converted the evolved sulphide R.S.R′ into the mercurichloride, mercuribromide, benzylsulphonium picrate and styphnate. Their melting points differed from those of corresponding diethyl sulphide derivatives, approximating to those of methyl-*n*-propyl sulphide. The derivatives were non-homogeneous. Mercurichloride analysis suggested

$$2CH_3.S.C_3H_7.5HgCl_2.$$

The sulphilimine, $R.R'.S \rightarrow N.SO_2.C_6H_4.Me$, on repeated crystallization had m.p. 103–104° and 104–105° mixed with methyl-*n*-propylsulphilimine, m.p. 105°.

The sulphide from dog's urine was converted to the methylsulphonium base (X) $[R.\overset{+}{S}.R'(Me)]\ \overset{-}{O}H$. This was chromatographed (acid-washed paper, butanol-CH_3COOH; bromophenol blue) simultaneously with mixtures of (*a*) $[Me_2\overset{+}{S}.n\text{-}Pr]\ \overset{-}{O}H$ with traces of (*b*) $[Me_2\overset{+}{S}.n\text{-}Bu]\ \overset{-}{O}H$ and (*c*) $[Me_2.\overset{+}{S}.iso\text{-}Bu]\ \overset{-}{O}H$, respectively. Pattern $(a+b)$ was identical with X, pattern $(a+c)$ was different.

The sulphilimine from dog's urine was then chromatographed (Perspex-coated paper, ethyl acetate; aqueous KI + HCl; iodine stain). Two spots appeared. Corresponding sections were removed, separately boiled with $NaHSO_3$ and the evolved sulphides converted into mercurichlorides. The main band gave m.p. 164–165° and mixed m.p. 165° with $2Me.S.n\text{-}Pr.5HgCl_2$, m.p. 165°. The minor band, m.p. 110–114°, sintering slightly at 90°, behaviour identical with that of Me.S.*n*-Bu mercurichloride.

The sulphide from dog's urine is therefore mainly methyl-*n*-propyl sulphide with some *n*-butylmethyl sulphide.

REFERENCES

Abel, J. J. (1895). *Hoppe-Seyl. Z.* **20**, 253.

Neuberg, C. & Grosser, P. (1905–6). *C. Bl. Physiol.* **19**, 316.

Enzymic Synthesis of Alkyl Esters of Ribomononucleotides. By L. A. HEPPEL and P. R. WHITFELD (introduced by R. MARKHAM). (*Agricultural Research Council Plant Virus Research Unit, Molteno Institute, Cambridge*)

Various alkyl esters of ribomononucleotides have been synthesized chemically, and it has been found that some of them are hydrolysed by pancreatic ribonuclease (Brown & Todd, 1953). We have now been able to demonstrate their enzymic synthesis by ribonuclease and by a fraction from spleen.

Ribonuclease catalyses the synthesis of cytidine methyl (or ethyl) phosphate from cytidine-2′:3′ phosphate and the appropriate alcohol. The identification of the product is based upon its R_F values in two solvents and ionophoretic mobility at pH 3·5, compared with the chemically synthesized ester (from Dr D. M. Brown). Further, its stability to 0·1 N-HCl corresponds to that of alkyl esters, in contrast to cytidine-2′:3′ phosphate which is more acid labile. The enzymic product is split during prolonged digestion with ribonuclease to give cytidylic acid. Uridine-2′:3′ phosphate participates in similar reactions.

Ribonuclease also catalyses the formation of cytidine methyl phosphate from cytidine-3′ benzyl phosphate and methanol. Cytidine-2′ benzyl phosphate is inactive, a result to be expected, since Brown & Todd (1953) found that only the 3′-ester is hydrolysed by ribonuclease. Paper chromatography of the product in ammonium sulphate-sodium acetate-*iso*propanol (Markham & Smith, 1952) shows only one spot, running in the position of one of the chemically synthesized isomers, and presumably is cytidine-3′ methyl phosphate.

Experiments with an enzyme fraction from spleen (cf. Heppel, Markham & Hilmoe, 1953) have also shown the formation of cytidine methyl phosphate from cytidine-3′ benzyl phosphate and methanol. In contrast to ribonuclease, however, spleen enzyme catalyses, in addition, the production of methyl and ethyl esters of adenylic acid from the corresponding benzyl ester.

In the above reactions one can demonstrate the nearly complete disappearance of the cyclic mononucleotide or of the benzyl ester and a substantial accumulation of the methyl ester with 1 M methanol and the other reactants present in a concentration of 0·01 M. The reactions proceed to a considerable extent with 0·25 M methanol.

REFERENCES

Brown, D. M. & Todd, A. R. (1953). *J. chem. Soc.* p. 2040.
Heppel, L. A., Markham, R. & Hilmoe, R. J. (1953). *Nature, Lond.*, **171**, 1151.
Markham, R. & Smith, J. D. (1952). *Biochem. J.* **52**, 552.

Exchange Reactions Involving Dinucleotides and Cyclic Mononucleotides.

By L. A. HEPPEL, P. R. WHITFELD and R. MARKHAM. (*Agricultural Research Council Plant Virus Research Unit, Molteno Institute, Cambridge*)

Pancreatic ribonuclease has been found to catalyse the formation of a dinucleoside monophosphate from a cyclic pyrimidine mononucleotide and a pyrimidine nucleoside. Two reactions of this type have been demonstrated:

(1) Dicytidine monophosphate is formed from cytidine-2′:3′ phosphate and cytidine.

(2) Uridylyl cytidine is formed from uridine-2′:3′ phosphate and cytidine.

With excess nucleoside a large fraction of the cyclic mononucleotide reacts to form the dinucleoside monophosphate. The identification of the product is based upon its R_F value in *iso*propanol-water-NH_3 and in ammonium sulphate-sodium acetate-*iso*propanol (Markham & Smith, 1952), a base:phosphorus ratio of 2:1, and the fact that further digestion of the product with ribonuclease yields equal quantities of mononucleotide and nucleoside. In the case of dicytidine monophosphate, the ionophoretic mobility of the enzymatic product at pH 3·5 is 0, whereas the starting materials have a considerable mobility in opposite directions. There is evidence that carbon 5′ of the nucleoside is esterified in forming the new diester link because the product is oxidized by periodate, and is also hydrolysed to nucleosides by snake venom. The enzymic product is also attacked by ribonuclease, and therefore carbon 3′ of the cyclic mononucleotide is involved in forming the phosphate bridge between the nucleoside residues.

With an enzyme fraction from spleen (cf. Heppel, Markham & Hilmoe, 1953) several exchange reactions have been demonstrated:

(1) Adenylyl uridylic acid + cytidine → adenylyl cytidine + uridylic acid.

(2) Adenylyl uridylic acid + methanol → adenosine methyl phosphate + uridylic acid.

Both of these reactions proceed to a considerable extent as written; their reversibility has not been investigated.

REFERENCES

Heppel, L. A., Markham, R. & Hilmoe, R. J. (1953). *Nature, Lond.*, **171**, 1151.
Markham, R. & Smith, J. D. (1952). *Biochem. J.* **52**, 552.

The Dephosphorylation of Thiamine Pyrophosphate by Suspensions of Cerebral Tissues.

By O. E. PRATT. (*Departments of Biochemistry and Neuropathology, Institute of Psychiatry, British Post-graduate Medical Federation, Maudsley Hospital, London, S.E.* 5)

Thiamine pyrophosphate is hydrolysed by brain and by other animal tissues (Peters, 1937; Ochoa, 1939; Westenbrink, Steyn Parvé & Goudsmit, 1943). Histochemical investigations into the localization of enzymes in brain showed that the histological sites of thiamine pyrophosphate hydrolysis did not correspond with the sites of glycerophosphate hydrolysis by the non-specific acid or alkaline phosphatases (Naidoo & Pratt, 1952, 1953). Confirmation of the presence of a separate enzyme has been provided by the following study.

Determinations were made of the rates of inorganic phosphate liberation, by fresh saline suspensions of whole rat brain, from glycerophosphate at pH 5·3 (acetate buffer) or pH 9·1 (veronal buffer), or from thiamine pyrophosphate at pH 6·9 (maleate buffer). The rate of thiamine pyrophosphate hydrolysis was maximal at pH 6·9, falling off more rapidly on the acid side than on the alkaline, whilst that of glycerophosphate was minimal at about pH 6·9. The optimal thiamine pyrophosphate concentration was 10^{-3} M. Considerable activation was produced by Mg^{2+} (0·03 M) or Ca^{2+} (0·05 M), the latter being used routinely. The acid phosphatase hydrolysing glycerophosphate differed in that a high proportion of the activity was obtained in the absence of added divalent cations.

There were differences in the inhibitor concentrations affecting the different reactions. Thus, the ratio $(\text{Inh.})_{50}$ thiamine pyrophosphatase/$(\text{Inh.})_{50}$ acid

phosphatase was 190 for NaF but only 0·1 for $La(NO_3)_3$. The ratio $(I)_{50}$ thiamine pyrophosphatase/$(I)_{50}$ alkaline phosphatase was 200 for cysteine. An acetone dried brain suspension showed almost no thiamine pyrophosphatase activity, although retaining the ability to hydrolyse glycerophosphate. A brain suspension prepared by grinding in veronal buffer (pH 9·1, 0·1 M) showed high thiamine pyrophosphatase activity but much reduced nonspecific acid phosphatase activity. Confirmation that thiamine pyrophosphate is hydrolysed by an enzyme that is distinct from the alkaline phosphatase is provided by the histological localization of the former mainly in nerve cell bodies and the latter mainly in blood capillaries. The differential effects of inhibitors or of acetone or alkali treatment also distinguish the thiamine pyrophosphatase from the acid phosphatase hydrolysing glycerophosphate.

These findings indicate that the dephosphorylation of thiamine pyrophosphate by brain is carried out by a separate enzyme.

REFERENCES

Naidoo, D. & Pratt, O. E. (1952). *J. Neurol. Psychiat.* **15**, 164.

Naidoo, D. & Pratt, O. E. (1953). *Enzymologia* (in the Press).

Ochoa, S. (1939). *Biochem. J.* **33**, 1262.

Peters, R. A. (1937). *Biochem. J.* **31**, 2240.

Westenbrink, H. G. K., Steyn Parvé, E. P. & Goudsmit, J. (1943). *Enzymologia*, **11**, 26.

The Distribution of Radioactivity in Goat Casein after Injection of Radioactive Amino Acids and its bearing on Theories of Protein Synthesis. By B. A. Askonas, P. N. Campbell and T. S. Work. (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

Four radioactive amino acids, [14C]glycine, [14C]-valine, [14C]lysine, and [35S]methionine, were injected simultaneously into a lactating goat and milk samples were collected at 4 and 8 hr. A 1 g. sample of the 4 hr. casein was hydrolysed for 18 hr. at 105° with 6N-HCl and a second sample for 12 hr. at 56° with conc. HCl. Five radioactive amino acids were isolated from the complete hydrolysate and had the following relative specific activities (counts/min./cm.^{2}): glycine 11400, serine 1510, valine 1008, lysine 3170, methionine 625. The partial hydrolysate was fractionated first on charcoal, then on buffered Dowex 50, and finally, after conversion of the peptides into dinitrophenyl-peptides, on buffered silica columns. The following peptides were isolated: leuc.gly., thre.[gly.glu.], thre.[asp.asp.val.], val.-[pro.leuc.val.], val.[?] (accidental loss of water-soluble fraction of DNP-peptide hydrolysate), lys.[asp.glu.leuc.phe.], lys.[glu.phe.lys.], ser.pro. and ser.leuc. Identification was provisional and depended on paper chromatography; the compound listed as a lysine pentapeptide may be a mixture of two lysine tripeptides. The sequence of amino acids enclosed by square brackets was not determined. Each peptide was hydrolysed, the radioactive amino acid isolated as the dinitrophenylamino/acid, and its relative specific activity determined. The four radioactive valines gave counts of 1120, 1040, 1680 and 1100; the three lysines gave counts of 2780, 3040 and 2950: two serines had counts of 1475 and 1334, and two glycines counts of 11200 and 10200. The amino acids isolated from different peptide sequences must come from different parts of the casein molecule but, except for one valine, all amino acids isolated from peptides had substantially the same radioactivity as the corresponding amino acid isolated from a complete hydrolysate of casein.

These results suggest that casein is synthesized from free amino acids and that no peptides are supplied for casein synthesis by partial breakdown of plasma protein. They do not exclude the possibility that peptide intermediates are formed during protein synthesis, but they indicate that any such peptide must have a transient existence.

Some Effects of Inorganic Phosphate and Bicarbonate on Cell Survival and Proliferation in Chemically Defined Nutrient Media. By Charity Waymouth. (*Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, U.S.A.*)

The common physiological salt solutions used as components of tissue culture media (Tyrode, 1910; Pannett & Compton, 1924; Gey & Gey, 1936; Earle, 1943; Hanks, 1948) contain amounts of inorganic phosphate of the order 0·4–1·0 mM. Simms & Sanders' (1942) solution X 6 for adult fibroblasts contains 1·5 mM, and the Z 16 solution designed for adult epithelium contains 9·0–12·0 mM phosphate (Parshley & Simms, 1950). Pannett & Compton's (1924) solution, and the high phosphate Z 16 solution of Parshley & Simms, contain no bicarbonate. The other salt solutions contain

bicarbonate at (Earle, 1943, 26·0 mM) physiological levels or lower (e.g. Tyrode, 1910, 12 mM; Hanks, 1948, 1·5 or 4·2 mM), and either monobasic or dibasic phosphate or both in a buffer of pH 7·4–7·8.

The synthetic nutrient solutions hitherto described (White, 1946; 1949; Morgan, Morton & Parker, 1950) have contained amounts of phosphate and bicarbonate based on the concentrations in balanced salt solutions designed to imitate blood plasma. The classical biological nutrient media contain extracts of embryonic tissue in addition to plasma or serum. The total phosphorus of chick-embryo extract (1:1) as usually prepared is 8·0–10·0 mM (Waymouth & Laurence, unpublished), or several times the amount provided in plasma or the usual salt solutions. It has been shown (Harris, 1952) that, in the presence of dialysed embryo extract, chick-embryo tissues exhibit a specific requirement for bicarbonate at about 25 mM, that 1·0 mM inorganic phosphate is adequate for growth in the presence of available organic phosphate and that 3·0 mM supplementary inorganic phosphate is inhibitory.

In the present study of the survival, maintenance of function and proliferative activity of cells in simple, chemically defined media, it has been found that these properties of the cells are all enhanced by increasing the amounts of inorganic phosphate in the synthetic media to about 2 mM. Inorganic P has proved to be a limiting factor for survival, for maintenance of heart-muscle contraction and of ciliary activity in lung epithelium. This suggests that one of the so-called 'growth factors' in embryo extracts is inorganic phosphate, or labile phosphate readily available. Besides contributing to the buffering capacity of the medium, the phosphate probably exercises an important function in maintaining the appropriate conditions for phosphorylation and the production of high-energy phosphate. In the presence of 26·6 mM glucose (480 mg./100 ml.) optimum concentrations of inorganic P and bicarbonate for several types of cell have been found to fall in the range 1·77–2·36 mM-P and 19·95–26·6 mM-$NaHCO_3$.

This work was done during the tenure of an American Cancer Society British-American Exchange Fellowship, recommended by the British Empire Cancer Campaign and the Committee on Growth of the National Research Council of the U.S.A. The author is grateful for the opportunities afforded by this Fellowship; to Prof. A. Haddow and the Board of the Royal Cancer Hospital, London, for leave of absence; and to Dr P. R. White for constant encouragement.

REFERENCES

Earle, W. R. (1943). *J. Nat. Cancer Inst.* **4**, 165.
Gey, G. O. & Gey, M. K. (1936). *Amer. J. Cancer*, **27**, 45.
Hanks, J. H. (1948). *J. cell. comp. Physiol.* **31**, 235.
Harris, M. (1952). *J. cell. comp. Physiol.* **40**, 279.
Morgan, J. F., Morton, H. J. & Parker, R. C. (1950). *Proc. Soc. exp. Biol., N.Y.*, **73**, 1.
Pannett, C. & Compton, A. (1924). *Lancet*, **206**, 381.
Parshley, M. S. & Simms, H. S. (1950). *Amer. J. Anat.* **86**, 163.
Simms, H. S. & Sanders, M. (1942). *Arch. Path.* **33**, 619.
Tyrode, M. V. (1910). *Arch. int. Pharmacodyn.* **20**, 206.
White, P. R. (1946). *Growth*, **10**, 231.
White, P. R. (1949). *J. cell. comp. Physiol.* **34**, 221.

Examination of the Effect of *Clostridium oedematiens* Toxin on Certain Enzyme Systems.

By Gillian M. Lewis and Marjorie G. Macfarlane. (*Lister Institute, London*)

Culture filtrates of *Clostridium oedematiens* injected into animals produce a gelatinous oedema and marked haemo-concentration. This suggested that the lethal factor, the α-toxin, might inhibit some enzyme system maintaining selective permeability. The action of the toxin *in vitro* on a number of respiratory and other enzymes was therefore examined. In complex systems, the effect of the toxin on each component (apo-enzyme, coenzyme, substrate) was examined in turn by making the particular component the rate-limiting factor in the reaction.

The toxin used, derived from *Cl. oedematiens*, type B, strain Albiston, contained 4000 (mouse) LD_{50}/mg. N; it had slight lecithinase activity, approx. equivalent to 0·24 LD_{50}/mg. N, but no other haemolytic activity. The enzyme or other component examined was incubated in small quantities with and without 500–1000 LD_{50} toxin at about neutral pH for 10–20 min. at 38°; the remaining components were then added and the relative enzymic activities with and without toxin compared.

No effect of the toxin was observed on any component of the following systems: (muscle) glycolysis of glycogen and glucose; Lohmann's enzyme, creatine, phosphocreatine; diphosphopyridine nucleotide-dependent dehydrogenases; (kidney) D-amino acid oxidase; (liver) acetylating enzyme and coenzyme A; catalase; (blood) acetylcholine esterases, carbonic anhydrase; (yeast) hexokinase, carboxylase; (milk) xanthine oxidase.

The oxygen uptake of a rabbit-muscle brei was inhibited 50% after preincubation with toxin (1000 LD_{50}/mg. fresh tissue); the cytochrome oxidase activity of rat-liver mitochondria was also reduced by the toxin in absence of added cytochrome, but not if cytochrome *c* were added. This inhibition, however, was not due to the lethal component (α-toxin) of the crude toxin, as it was not prevented by pre-neutralization with the specific antitoxin. The lecithinase activity of the toxin was insufficient to account for this effect. Oxidative phosphorylation by rat mitochondria in presence of L-glutamate, adenylic acid and added cytochrome *c* was not affected by the toxin.

The possibility that the lethal toxin is present in the culture filtrates as a 'protoxin', activated *in vivo* by tissue constituents, was offset to some extent by the use of crude tissue enzyme preparations; addition of boiled tissue extracts or cysteine had also no activating effect. Though the possibility of false negative results cannot be completely excluded it seems unlikely that the *Cl. oedematiens* lethal toxin directly affects the energy-producing systems of tissues.

Effects of Adrenal Corticoids on Lipogenesis in Mammary Tissue *in vitro*. By JUDITH H. BALMAIN, S. J. FOLLEY, R. F. GLASCOCK and MARY L. McNAUGHT. (*National Institute for Research in Dairying, University of Reading*)

We have shown that cortisone, *in vitro* at high concentrations, inhibits lipogenesis by mammary-gland slices from lactating rats and partially blocks the stimulatory effect of insulin on this process (Balmain, Folley & Glascock, 1952). The effects of adrenal corticoids on the incorporation of glucose and acetate carbon into fatty acids by lactating mammary slices from the rat and ewe have been further studied. In some cases ^{14}C and ^{3}H were assayed by gas counting (Glascock, 1952); in others ^{14}C only has so far been assayed by end-window counting.

In rat slices (substrate: [^{14}C]glucose + [Me-^{3}H]-acetate) cortisone (approx. 100 μg./ml.) decreased the incorporation of acetate and glucose carbon, confirming our previous finding. Incorporation of glucose carbon was also strongly inhibited by deoxycorticosterone (100 μg./ml. but not 50 μg./ml.) and corticosterone (100 μg./ml.). On the other hand, 17-hydroxycorticosterone (100 μg./ml.) had no inhibitory effect on the incorporation of glucose or acetate carbon and did not antagonize the effect of insulin. The results for sheep slices were somewhat different. Cortisone (100 μg./ml.) had no consistent effect either way on the incorporation of acetate or glucose carbon (substrate: [^{14}C]glucose + [Me-^{3}H]acetate in some experiments; [1-^{14}C]acetate + glucose in others); nor had 17-hydroxycorticosterone (100 μg./ml.) any consistent effect on acetate carbon uptake (substrate: [1-^{14}C]acetate + glucose). Deoxycorticosterone and corticosterone (100 μg./ml. but not 50 μg./ml.) both strongly inhibited the uptake of acetate carbon (substrate: [1-^{14}C]acetate + glucose).

The absence of an inhibitory effect of 17-hydroxycorticosterone on lipogenesis in rat mammary slices by contrast with the marked inhibitory effect of equal concentrations of all other corticoids studied, whether oxygenated at $C_{(11)}$ or not, is of interest in view of the possibility that 17-hydroxycorticosterone may be the natural hormone of the adrenal cortex. This may not be so in the rat, for Bush (1953) reports that rat adrenal vein blood contains corticosterone and 17-hydroxycorticosterone with the former greatly predominating. In the rat some control of lipogenesis could perhaps be exercised through changes in the proportion of these two steroids secreted by the adrenals. The different response of sheep udder tissue to cortisone is of interest in connexion with previously observed metabolic differences *in vitro* between mammary tissue from ruminants and non-ruminants (Balmain, Folley & Glascock, 1953).

We are indebted to Dr C. C. Porter, of the Merck Institute for Therapeutic Research, for cortisone (free alcohol), to Dr A. White, formerly of Chemical Specialities Co. Inc., for corticosterone and 17-hydroxycorticosterone, and to Dr W. J. Tindall of Organon Laboratories Ltd. for deoxycorticosterone.

REFERENCES

Balmain, J. H., Folley, S. J. & Glascock, R. F. (1952). *Nature, Lond.*, **169**, 447.
Balmain, J. H., Folley, S. J. & Glascock, R. F. (1953). *Biochem. J.* **53**, xxvi.
Bush, I. E. (1953). *Recent Progr. Hormone Res.* **8**, 264.
Glascock, R. F. (1952). *Biochem. J.* **52**, 699.

The Effect of an Anti-thyroxine Compound on the Deiodination of Triiodothyronine in Rats.

By J. H. Wilkinson and N. F. Maclagan. (*Department of Chemical Pathology, Westminster Medical School, London, W.S.* 1)

In an attempt to explain the metabolic effects of the anti-thyroxine compound, butyl 4-hydroxy-3:5-diiodobenzoate (BHDB), in mice, Maclagan, Sprott & Wilkinson (1952) suggested that BHDB might act by inhibiting deiodination processes. If in the ordinary course of metabolism, thyroxine undergoes conversion into triiodothyronine and the latter is further deiodinated to inactive substances, then interference with these processes would lead to diminution of the effects of thyroxine and enhancement of those of triiodothyronine, as actually observed.

The first part of this hypothesis was tested and BHDB was shown to reduce the extent of iodide excretion in the urine of rats after treatment with thyroxine (Wilkinson & Maclagan, 1953). It remains to ascertain whether the drug has a similar effect when triiodothyronine is given.

Triiodothyronine labelled in the 3′-position with ^{131}I was given subcutaneously in a dosage of 6 μg./rat to four groups of three rats (body wt. 140 ± 15 g.). Two of the groups were also given 50 mg./rat BHDB orally on the previous day, and 25 mg./rat daily throughout the period of the experiment. The remaining two groups received the medium (1% methoxycarbonylcellulose) in place of BHDB. Each group was kept in a metabolism cage and the urine and faeces were collected daily. The urinary and faecal radioactivity were measured in a Veall liquid counter. A similar experiment was performed in which triiodothyronine was given at a dosage of 30 μg./rat.

The percentages of the dose excreted in the urine during 5 days by the eight groups of rats were: triiodothyronine (6 μg./rat), 25·9, 25·0; triiodothyronine (6 μg./rat) + BHDB (50 and 25 mg./rat daily), 8·5, 13·4; triiodothyronine (30 μg./rat), 20·6, 20·1; triiodothyronine (30 μg./rat) + BHDB (50 and 25 mg./rat daily), 9·0, 10·5.

The combined urinary and faecal excretion of all groups over the 5-day period was 65–80% of the injected dose.

Since the urinary radioactivity has been shown to consist of iodide and not thyronine derivatives (Wilkinson & Maclagan, 1953), it is clear that BHDB exercises a marked restraint on the deiodination of triiodothyronine. The results therefore support our hypothesis as to the mode of action of BHDB.

REFERENCES

Maclagan, N. F., Sprott, W. E. & Wilkinson, J. H. (1952). *Lancet*, ii, 915.

Wilkinson, J. H. & Maclagan, N. F. (1953). *J. Endocrin.* (in the Press).

The Detection of Thyroxine and Related Substances on Paper Chromatograms by the Ceric Sulphate-Arsenious Acid Reaction.

By C. H. Bowden and N. F. Maclagan. (*Department of Chemical Pathology, Westminster Medical School,* 17 *Horseferry Road, London, S.W.* 1)

In the course of work on the urinary excretion of anti-thyroxine compounds (Maclagan & Wilkinson, 1951; Fraser & Maclagan, 1953), it was noted that the ceric sulphate-arsenious acid test for iodides was also applicable to certain iodine-containing organic compounds. The application of this method to the demonstration of thyroxine and related substances on paper chromatograms was therefore studied. Preliminary attempts in which the reagent was sprayed on the paper were unsuccessful, but after many trials satisfactory results were obtained by saturating a second blank paper with the reagent and then applying the chromatogram to this between glass slides. The detailed technique was as follows:

Equal volumes of 10% (w/v) $Ce(SO_4)_2.4H_2O$ in N-H_2SO_4 and 5% (w/v) sodium arsenite in N-H_2SO_4 are mixed and evenly applied to a no. 1 Whatman chromatographic paper strip of the same size as the chromatogram, by laying the paper flat on a sloping clean glass plate and allowing the reagent to soak the paper. The dry chromatogram is then placed on the wet sheet and another glass plate placed on top and pressed firmly down. After development for 30 min. both papers are dried in an iodine-free atmosphere.

The iodine-containing compounds are indicated by white spots on a yellow background. The chromatogram when dry is only stable for 1–2 days, but permanent records can be made either by direct photography with a blue filter or by backing the chromatogram with photographic paper followed by exposure to ultraviolet light. Paper stained with ceric sulphate interrupts the transmission to ultra-

violet light, whereas the white spots do not. Alternatively, the paper may be interposed between the ultraviolet lamp and a photoelectric cell, as described by Nanninga & Bink (1951) in a different connexion, and the resulting current measured. In this method the lead-glass screen recommended by these workers is omitted.

The limiting quantities detectable with typical compounds by the various methods of recording after chromatography for 16 hr. in a butanol-dioxan-ammonia system at room temperature were determined. It was found that the sensitivity is many times greater than that of previous methods for these compounds. By the photoelectric method semi-quantitative results can be obtained in the range from 0·01 to 0·1 μg. for KI and from 0·1 to 1 μg. for thyroxine or triiodothyronine. The method is applicable to a wide range of organic and inorganic iodine compounds.

REFERENCES

Fraser, T. R. & Maclagan, N. F. (1953). *J. Endocrin.* **9**, 301.
Maclagan, N. F. & Wilkinson, J. H. (1951). *Nature, Lond.*, **168**, 251.
Nanninga, L. & Bink, B. (1951). *Nature, Lond.*, **168**, 389.

Fractionation of the Intracellular Material of *Bacillus anthracis* Grown *in vivo*; the Polysaccharide and Polyglutamic Acid. By H. Smith and H. T. Zwartouw. (*Microbiological Research Department, Porton, Wilts*)

Bacillus anthracis isolated from infected guinea pigs, and differing from organisms cultured *in vitro*, lysed in $(NH_4)_2CO_3$ solution (0·16 % w/v) at 0°. The solution (pH 8·5) lost all $(NH_4)_2CO_3$ on freeze-drying (Smith, Keppie & Stanley, 1953*a*, *b*).

Dialysis removed 50 % of the material, including solids from the Locke's solution in the original bacterial sludge (Smith *et al.* 1953*b*). The dialysate (1·6 % w/v) was fractionated at pH 7. The fractions and their essential nature were as follows: (1) barium acetate (1 % w/v) added at 20° (yield 7 %, protein); (2) ethanol added to 5 % v/v at 0° (yield 8 %, polyglutamic acid); (3) ethanol added to 33 % v/v at 0° (yield 14 %, protein); (4) material remaining soluble (yield 9 %, polysaccharide). Each fraction was treated with Na_2SO_4 to precipitate barium, centrifuged, dialysed and freeze-dried.

The polysaccharide (1 % w/v) in sodium acetate solution (1 % w/v) was fractionated with ethanol (50–70 % v/v). Fuller's earth powder removed small amounts of bacterial protein. The sample was ultracentrifugally and electrophoretically homogeneous at pH's 4 and 8 and modified partial solubility studies gave no evidence for heterogeneity. It had galactose (anthrone method), 43·0 % (± 2); hexosamine, 37·0 % (± 2); N, 3·95; distillable acid after hydrolysis calculated as acetyl, 14·7 %; $[\alpha]_D^{20} + 81° \pm 5°$. Paper chromatography showed galactose, glucosamine, and ninhydrin positive spots different from those due to glucosamine and equivalent to the amino acids in 4 % of bacterial protein. Repeated treatment with fuller's earth, which removed bacterial protein completely from solution, has not reduced the N or amino acid figure. This analysis is different from that of a polysaccharide extracted by more drastic methods from *B. anthracis* cultured *in vitro* (Ivanovics, 1940). A dilution (1–640000) of the polysaccharide precipitated serum provided by the Instituto Seiroteripico Milanese Serafino Balfanti for the Ascoli thermo-precipitation test.

Polyglutamic acid was purified by refractionating with barium acetate and ethanol and treatment with fuller's earth powder. It was electrophoretically and ultracentrifugally homogeneous at pH's 4 and 8, and had N, 9·0; sulphated ash, 44·5; foreign amino acids, nil (less than those in 0·2 % of bacterial or plasma protein); carbohydrate, nil; $(C_5H_6O_3NNa)_n$ requires N, 9·3; sulphated ash, 47·0 %.

Some properties of the protein fractions are described in the following abstract.

Acknowledgement is made to the Chief Scientist, Ministry of Supply, for permission to make this communication.

REFERENCES

Ivanovics, G. (1940). *Z. ImmunForsch.* **97**, 402.
Smith, H., Keppie, J. & Stanley, J. L. (1953*a*). *Brit. J. exp. Path.* (in the Press).
Smith, H., Keppie, J. & Stanley, J. L. (1953*b*). *Brit. J. exp. Path.* (in the Press).

The Nature of the Aggressins Produced by *Bacillus anthracis* Growing *in vivo*. By H. Smith, H. T. Zwartouw and R. C. Gallop. (*Microbiological Research Department, Porton, Wilts*)

Keppie, Smith & Harris-Smith (1953) have shown that *Bacillus anthracis* when growing in guinea pigs produces aggressins intracellularly, and also extracellularly in the body fluids of the host. Both products have now been fractionated. Aggressin activity has been measured by the capacity to interfere with the phagocytosis of virulent *B. anthracis* (grown in broth) by guinea-pig phagocytes (Keppie *et al.* 1953). The concentrations (w/v) of fractions used in this test are given in brackets.

Of the intracellular products (see previous abstract) diffusate (2·5 %) and the polysaccharide (2·5 %) were inactive, while polyglutamic acid was active at high concentrations (2 %). High activity was found in the protein fractions (0·025 %), which after refractionation with barium acetate and ethanol were free from polyglutamic acid and the polysaccharide.

Extracellular products were separated efficiently from plasma protein in the body fluids by barium acetate/ethanol fractionation. Freeze-dried material in water (8 % w/v) at pH 7 and 0°, was dialysed (diffusate, yield 50 %, inactive (1·6 %)) and treated with barium acetate (2 % w/v). Fractions were taken at the following ethanol concentrations, and recovered as described in the previous abstract. (i) 5 % ethanol, yield 1 %, containing 60–70 % polyglutamic acid. When purified as described for the polyglutamic acid in the previous abstract it had the same criteria of purity and was active at high concentrations (2 %). (ii) 10 % ethanol, yield 1 %. After refractionation it had N, 13·2 %; ash, 2·8 %; all the usual amino acids; polyglutamic acid, nil; nucleic acid, nil; carbohydrate (Molisch), 1·5 %; hexosamine, 1·5 %; and was highly active (0·01 %). (iii) 20 % ethanol, yield 3 %, small activity (0·2 %). (iv) Soluble material, yield 35 %, inactive (3·2 %).

Polyglutamic acid, concentrated in the capsule surrounding *B. anthracis* may help to prevent phagocytosis, but the most powerful aggressin resides in highly active, essentially protein fractions isolated from inside and outside the cell. The latter have received little attention in previous chemical studies on *B. anthracis*.

Parallel tests for protective antigen (Keppie *et al.* 1953) on the fractions from the body fluids showed diffusate inactive (12·8 %), polyglutamic acid inactive (0·8 %), 10 % fraction highly active (0·025 %), 20 % fraction slightly active (0·2 %), and soluble material inactive (3·2 %). Concentration of aggressive and protective activity in the same fraction is noteworthy.

Acknowledgement is made to the Chief Scientist, Ministry of Supply, for permission to make this communication.

REFERENCE

Keppie, J., Smith, H. & Harris-Smith, P. W. (1953). *Brit. J. exp. Path.* (in the Press).

The Effect of Plasma β-Lipoprotein Concentration on the Action of Injected Heparin in the Rabbit. By A. Comfort.* (*Department of Zoology, University College, London, W.C. 1*)

Intravenous injection of heparin in rabbits made lipaemic by lanolin feeding produces a large increase in the anodic mobility of the β-lipoprotein front at pH 8·6. The same effect is produced *in vitro* by addition of plasma from heparin-injected animals, but not by heparin itself. It is therefore presumably due to heparin 'clearing factor' (Comfort, 1953).

We have followed the response of rabbits to injected heparin (*a*) during the decline of lipaemia after cessation of several months' lanolin feeding, (*b*) during an acute episode of lipaemia produced by one week of lanolin feeding. Blood was drawn before and 10 min. after injection of 1000 units of heparin (Pularin Evans) into the ear vein in young does (2–3 kg.), and examined by paper electrophoresis and staining with Sudan black B, as previously described by us. Serum cholesterol was estimated in duplicate on the pre-injection sample by the method of Abell, Levy, Brodie & Kendall (1952).

At serum cholesterol levels > 1 mg./ml. heparin produced an increase in β-lipoprotein migration distance in all cases. The percentage increase varied greatly, both between animals, and in the same animal on different occasions. The magnitude of the increase bore no simple relation to blood cholesterol level or to dose/body weight.

At serum cholesterol levels < 1 mg./ml. approx., injected heparin either failed to affect β-lipoprotein mobility at all, or reduced it compared with parallel

* With a grant from the Nuffield Foundation.

controls. A very small increase in mobility could usually be produced at these levels by adding guinea-pig 'clearing factor', but not by increasing the dose of heparin. The slight action of heparin on the α_2-lipoprotein did not appear to be affected. The effect is not due to failure of 'clearing factor' production in the rabbit at low serum lipoid levels, since the heparinized plasma samples were active when tested against lipaemic rabbit plasma. The appearance of the electrophoretic strips suggests rather that this is a concentration effect than that the changing behaviour of rabbit lipoproteins with increasing lipaemia is due to the appearance of a heparin-sensitive component.

REFERENCES

Abell, L. L., Levy, B. B., Brodie, B. B. & Kendall, F. E. (1952). *J. biol. Chem.* **195**, 357.
Comfort, A. (1953). *Biochem. J.* **54**, xxiii.

Antithyroid Activity of some *S*-Substituted 2-Mercaptoglyoxalines. By A. LAWSON and C. E. SEARLE.* (*Royal Free Hospital School of Medicine, 8 Hunter Street, London, W.C.* 1)

Antithyroid drugs widely used in recent years for the treatment of hyperthyroidism have been 2-thiouracil, its 6-methyl and 6-propyl derivatives, and 2-mercapto-1-methylglyoxaline.

These substances are usually assumed to owe their activity to their rapid reduction of iodine to iodide, this process interfering with the iodination of tyrosine in the thyroid protein and hence with the synthesis of the thyroid hormone. A free —SH group has therefore been regarded as essential. 2-Carbethoxythio-1-methylglyoxaline was however shown to have antithyroid activity in the rat comparable to that of the parent thiol (Lawson, Rimington & Searle, 1951) and has since been used clinically (Doniach, 1953; Poate, 1953).

This substance probably owes its activity to an *in vivo* hydrolysis to the active thiol, and some similar compounds, 2-carbomethoxythio-, 2-carbobenzyloxythio- and 2-hippurylthio-1-methylglyoxaline, and di(1-methylglyoxaline) 2:2-dithiolcarbonate, all show high activity in the rat.

It had been expected that 2-alkylthioglyoxalines would be quite devoid of activity, but some slight activity had been observed in 2-benzylthioglyoxaline (Searle, Lawson & Hemmings, 1950). Some *S*-benzyl- and *S*-methyl-derivatives of active 2-mercaptoglyoxalines were therefore examined by the same method but at rather larger dosages (25–50 mg./kg. body weight), and in several cases over 50% depressions of radio-iodine uptake over 4 hr. were observed. 2-Benzylthio-4-methylglyoxaline in this test appears to be about one tenth as active as 2-mercapto-4-methylglyoxaline itself.

Whilst the possibility exists that some thiol may be produced in the body by removal of the methyl or benzyl groups, the usual result of *in vitro* reductions is fission of the glyoxaline-sulphur bond. Since from the action of iodine on 2-benzylthio-4-methylglyoxaline a 5-iodo-derivative has been obtained, *in vivo* iodination may account for the activity of these compounds.

* I.C.I. Research Fellow.

REFERENCES

Doniach, D. (1953). *Lancet*, i, 873.
Lawson, A., Rimington, C. & Searle, C. E. (1951). *Lancet*, ii, 619.
Poate, H. (1953). *Lancet*, i, 879.
Searle, C. E., Lawson, A. & Hemmings, A. W. (1950). *Biochem. J.* **47**, 77.