PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **309th Meeting** *of the Biochemical Society was held in the School of Biochemistry, Tennis Court Road, Cambridge, on Saturday,* 21 *June* 1952, *when the following papers were read.*

COMMUNICATIONS

The Dialuric Acid Haemolysis Test for Vitamin E Deficiency in Rats. By DOROTHY H. HEARD (*Department of Pathology, University of Cambridge*), T. MOORE and I. M. SHARMAN (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*)

György & Rose (1949) found that the red blood cells of rats deficient in vitamin E became haemolysed when incubated with a dilute solution of dialuric acid. This abnormality could be corrected by giving the animals adequate doses of α-tocopherol, or even by the addition of tocopherol *in vitro*.

We have confirmed the validity of this test on numerous rats which were restricted to a basal diet deficient in vitamin E, and either dosed or not dosed with α-tocopherol. Although albino and piebald rats show slight differences in their response to vitamin E deficiency (Moore, 1950) the corpuscles of both breeds were liable to haemolysis. Corpuscles from rats deficient in vitamins A or D, but adequate in vitamin E, were not haemolysed. Sometimes haemolysis was observed, however, in corpuscles from rats which had been given diets containing 20 % of cod liver oil in conjunction with doses of tocopherol which would otherwise have been adequate. The action of cod liver oil in increasing the requirement for vitamin E is well known. Corpuscles from rats given diets deficient in protein usually resisted haemolysis, but one exception was observed in a rat which had been deficient for over a year, and which was later found to have a fatty liver.

Dam and his colleagues have reported that rats may be protected against most of the effects of vitamin E deficiency by several substances which are not chemically related to the vitamin, including methylene blue. In the haemolysis test Christensen & Dam (1951) found that the dye gave marked but not complete protection. When methylene blue was added to our usual vitamin E-deficient diet, which differs considerably from those used by Dam, there was no protection against haemolysis. Some evidence of a vitamin E synergistic action, however, was seen in the delay of dental depigmentation and of brown discoloration of the uterus.

Examinations in a few human subjects with haemolytic diseases or in calves, under the care of Dr K. L. Blaxter, which had been fed upon excessive amounts of cod liver oil, gave no evidence that the corpuscles were sensitive to haemolysis.

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The Effects of Deoxyribonucleic Acid and Suramin on β-Glucuronidase Activity. By EVELYN E. B. SMITH and G. T. MILLS. (*Biochemistry Department, University of Glasgow*)

Bernfeld & Fishman (1950) have shown that dilution of a purified calf spleen β-glucuronidase preparation causes a decrease in the enzyme activity to protein ratio, and that there is present in boiled enzyme preparations a material which prevents this effect. They postulated that β-glucuronidase requires a coenzyme for full activity, and that this coenzyme dissociates on dilution. They showed that deoxyribonucleic acid (DNA) activates β-glucuronidase at pH 4·5, and also that DNA appears to fulfil the requirements of the postulated coenzyme of calf spleen β-glucuronidase.

In the present work it has been found that dilution of highly purified ox liver and spleen β-glucuronidase causes no decrease in the enzyme activity to protein ratio as found by Bernfeld & Fishman (1950). The activating effect of DNA at pH 4·5 has been confirmed, but it has been found that DNA causes an inhibition of purified ox liver β-glucuronidase at lower pH values. The relation of inhibition

to pH follows an S-shaped curve similar to that obtained for suramin inhibition of β-glucuronidase. The inhibition-pH curves are not identical for these two compounds, the point of 50% inhibition being pH 4·0 for DNA and pH 4·7 for suramin. The effects of DNA on β-glucuronidase are probably explicable on the basis of DNA being a polyacidic compound acting in like manner to suramin.

Some small molecular weight aromatic acids have been studied for their inhibitory effects on β-glucuronidase and these also exhibit an S-shaped pH-inhibition curve.

It is concluded from this work that there is no evidence to substantiate the claim of Bernfeld & Fishman (1950) that DNA is the coenzyme of β-glucuronidase. In addition, it is suggested that the theory of Wills & Wormall (1950) and Wills (1952), that the pH of 50% inhibition of an enzyme by suramin is the isoelectric point of the enzyme, may not apply in the case of β-glucuronidase.

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The Partition of Carotenoids and Vitamin A in the Milk of Cows and Goats throughout Lactation. By R. CHANDA. (*The Hannah Dairy Research Institute, Kirkhill, Ayr*)

Using chromatographic and spectrophotometric methods (Chanda, Owen & Cramond, 1951; Chanda & Owen, 1952) the partition of carotenoids and vitamin A in the milk of three cows from the 2nd to the 40th week of lactation and the partition of vitamin A in the milk of two goats from the 2nd to the 30th week of lactation were determined. All the animals were receiving winter rations. In cow milk fat the vitamin A content decreased gradually up to the 30th week. Thereafter, when the yield of milk was decreasing rapidly, both the percentage of fat in the milk and the percentage of vitamin A in the fat showed small but definite increases. A quadratic curve fitted to the data showed a minimum concentration of vitamin A in the fat at 25 weeks *post partum*. In goat milk fat the minimum occurred at 20 weeks.

In goat milk fat, 98% of the vitamin A was present as ester, vitamin A alcohol not being measurable. In cow milk, 5–8 i.u. vitamin A alcohol/100 ml. were always present, and the amount was unaffected by the stage of lactation. Carotenoids, though found in colostrum (Chanda & Owen, 1952), were not present in goat milk at any stage of lactation.

The carotenoid content of cow milk showed no changes as lactation advanced. Between the 2nd and 40th week of lactation, the amount of β-carotene present in cow milk fat ranged from 4·3 to 6·0 μg./g. fat; the mean was 5·1 μg./g. fat, and its coefficient of variation ±5·6%. Of the total carotenoids, β-carotene formed 65–85%, but there was evidence that the percentage was influenced by intake. Variations of the ratio of vitamin A to carotene were almost entirely determined by variations in the vitamin A content, thus presenting the reverse of the conditions found in the blood serum by Goodwin & Wilson (1951).

Goat milk contained 60 i.u. vitamin A/g. fat in the 2nd week of lactation. The corresponding figure for cow milk fat was 49 i.u. (vitamin A + β-carotene × 1·67). This superiority of the goat milk fat over that of the cow persisted throughout lactation, a fact which can perhaps be related to the greater activity of the thyroid gland of the goat which facilitates absorption of carotene and its conversion to vitamin A (Chanda, Clapham, McNaught & Owen, 1951; Chanda & Owen, 1952), and thus makes more of the vitamin available for secretion into the milk.

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The Amino-acid Sequence in the Glycyl Chain of Insulin. By F. SANGER and E. O. P. THOMPSON. (*Biochemical Laboratory, University of Cambridge*)

By oxidation of insulin with performic acid it is possible to split the S—S bridges, and separate two types of polypeptide chain in which the cystine residues have been converted into cysteic acid residues (Sanger, 1949). The amino-acid sequence in the chains with *N*-terminal phenylalanyl residues has previously been reported (Sanger & Tuppy, 1951). The fraction bearing glycyl *N*-terminal residues (fraction *A*) was hydrolysed with 11 N-HCl at 37° for 2–3 days, and the resulting peptides fractionated by paper chromatography after preliminary group separations using adsorption on charcoal and ionophoresis in silica gel or filter paper (Durrum, 1950).

On ionophoresis in 0·2 M-acetic acid peptides containing one cysteic acid residue remained as an approximately neutral band and could be completely separated from other peptides which moved towards the cathode. Those containing more than one cysteic acid residue moved towards the anode and could be identified as discreet bands.

The *N*-terminal residues of the peptides eluted from the chromatograms were determined by the dinitrophenyl (DNP-) method, the reaction being carried out in the presence of trimethylamine which could later be removed *in vacuo*. From the results with the peptides identified in the acid hydrolysate the following sequences were deduced as being present:

Gly . Ileu . Val . Glu . Glu . $CySO_3H$. $CySO_3H$. Ala (*N*-terminal sequence).

Ser . Leu . Tyr . Glu . Leu . Glu . Asp . Tyr . $CySO_3H$.

Ser . Val . $CySO_3H$.

$CySO_3H$. Asp.

When fraction *A* was treated with pepsin, a peptide was split off which contained no aspartic acid or tyrosine. On partial hydrolysis it gave rise to Ser . Val . $CySO_3H$ and Ser . Leu in addition to peptides from the *N*-terminal sequence. From these results it was deduced that the Ser . Val . $CySO_3H$ sequence adjoins the alanine residue in the intact polypeptide chain and the complete amino-acid sequence of the glycyl chain is therefore:

Gly . Ileu . Val . Glu . Glu . CyS . CyS . Ala . Ser . Val . CyS . Ser . Leu . Tyr . Glu . Leu . Glu . Asp . Tyr . CyS . Asp.

A number of other peptides were identified in the peptic hydrolysate and in hydrolysates obtained by the action of chymotrypsin, papain and the mould protease of Crewther & Lennox (1950). These peptides confirmed the above sequence. $CySO_3H$. Asp($—NH_2$) was liberated by the action of chymotrypsin, indicating that the *C*-terminal residue is asparagine. Carboxypeptidase (previously incubated with di*iso*propyl fluorophosphonate to inhibit the small amount of chymotrypsin impurity) liberates free asparagine from the *A* chain but only in small amounts. This is possibly due to inhibition from the adjacent cysteic acid group. Insulin treated with carboxypeptidase yields alanine (from the *B* chains; Lens, 1949) and also an appreciable amount of asparagine. Higher yields have been obtained using acetyl insulin and an approximate estimation by paper chromatography indicated 90 % liberation of the asparagine residues.

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Further Observations on the Amide and Free Carboxyl Groups of Insulin. By A. C. CHIBNALL and M. W. REES. (*Biochemical Laboratory, University of Cambridge*)

When the hydrolysate of reduced insulin (Chibnall & Rees, 1951) was subjected to two-dimensional chromatography (phenol-0·3 % NH_3; butanol-acetic acid), no evidence was found for the presence of γ-hydroxy-α-amino-*n*-butyric acid or its lactone, indicating the absence of aspartyl residues in the insulin molecule. Meanwhile Sanger & Thompson (see preceding communication) had found evidence for the presence of a *C*-terminal asparagine residue. We have accordingly re-investigated the esterification of this protein by treatment with diazomethane, and, as an alternative procedure, have also used that of Mommaerts & Neurath (1950). On reduction of the ester with $LiBH_4$, we have now obtained the results given in Table 1 which suggest that the insulin molecule (mol.wt. 11700) contains six glutaminyl, eight glutamyl and four asparaginyl residues located in the peptide chains. Two amide

groups must therefore be present as *C*-terminal residues.

Table 1. *Groups per molecule after hydrolysis*

	Insulin	Reduced insulin	Reduced 'oxidized' insulin (Sanger, 1949)
Glutamic acid	14	6	6
Aspartic acid	6	4	4
Total	20	10	10
Amide-NH_3	12	12	12

A *C*-terminal asparagine residue would appear in the reduced insulin hydrolysate as γ-hydroxy-β-amino-*n*-butyric acid or its lactone; these latter products have accordingly been synthesized. Both are retained on a column of Dowex-2 and can thus be separated from β-amino alcohols, which pass through it. Periodate analysis of a reduced insulin hydrolysate fractionated in this way showed the presence of two equivalents of the hydroxyamino-acid and two of alaninol. The β-amino alcohol fraction, nevertheless, gave two equivalents of formaldehyde in excess of those derived from the alaninol. Chromatography and oxidation to the amino-acid showed that this excess was due to the presence of approximately one equivalent of amino-ethanol with a subsidiary amount of leucinol.

When oxidized insulin (Sanger, 1949) was treated in a similar way a corresponding two equivalents excess of formaldehyde were obtained. Neither the glycyl nor phenylalanyl chain gave any such excess, and we confirm the presence in these of a *C*-terminal asparagine and alanine residue respectively (Sanger & Tuppy, 1951; Sanger & Thompson, 1952).

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Determination of the Amino-acid Sequence in Peptides. By J. Leggett Bailey (introduced by A. C. Chibnall). (*Biochemical Laboratory, University of Cambridge*)

The use of lithium aluminium hydride in the preparation of amino alcohols from amino-acid esters was first demonstrated by Karrer, Portmann & Suter (1948). Investigation of the reduction of peptide esters with this reagent has provided evidence of peptide carbonyl reduction. Use of lithium borohydride, as described by Chibnall & Rees (1951), produced intermediate boron complexes not easily split under mild conditions. It has been found that aluminium hydride exhibits a reactivity towards the carbonyl group intermediate between those of the two complex hydrides, and it has now been employed as a selective reducing agent for peptide esters. Reduction carried out at −40° in the medium aluminium chloride-tetrahydrofuran leads to an 85% conversion of the peptide ester to a β-hydroxyalkylamide.

In the degradative procedure under study the β-hydroxyalkylamide of the *N*-*p*-tosyl derivative of a peptide is the starting point. In the presence of such reagents as thionyl chloride or a phosphorus oxyhalide, a rearrangement takes place to give a β-amino ester (cf. Bergmann & Brand, 1923). The latter is then capable of a further treatment with aluminium hydride to yield the free amino alcohol and the residual peptide in a form ready for a further rearrangement:

$$\mathrm{RCONHCHR'CONHCHR''COOEt} \xrightarrow{\mathrm{AlH_3}} \mathrm{RCONHCHR'CONHCHR''CH_2OH} \xrightarrow{\mathrm{POCl_3}}$$

$$\mathrm{RCONHCHR'COOCH_2CHR''NH_2.HCl} \xrightarrow{\mathrm{AlH_3}} \mathrm{RCONHCHR'CH_2OH + HOCH_2CHR''NH_2}.$$

In the case of the hydroxyamide of an *N*-*p*-tosyl amino-acid an 80–85% rearrangement is possible. Preliminary experiments with the hydroxyamide of an *N*-*p*-tosyl dipeptide have indicated slightly less reactivity; a 60% conversion only to the *O*-peptidyl form has so far been achieved. Yields in excess of 90% have been obtained for the liberation of amino alcohols by reductive cleavage of the β-amino ester hydrochlorides employing aluminium hydride. Ion-exchange resins have facilitated the removal of inorganic residues resulting from hydrolysis of the metal hydrides.

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7-Dehydrocholesterol in the Sexual Organs of the Rat. By T. Moore and R. J. Ward. (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*)

The detection in extracts of certain regions of the male rat genital system of prominent absorption bands at 293, 282, 271 and 262 mμ., presumably indicative of the presence of 7-dehydrocholesterol, has already been mentioned (Moore & Ward, 1951). Further experiments have confirmed that this substance is present in the preputial glands, *caput epididymis* and *corpus epididymis*, in descending order of concentration, but not in the testes, *cauda epididymis*, seminal vesicles, coagulating glands and prostates. No selective absorption was seen in the unsaponifiable matter of brain extracts.

The *bulbi vestibuli* of the female, corresponding to the preputial gland in the male, sometimes contained 7-dehydrocholesterol, but none was detected in the ovaries, oviducts and uterus. In wild rats the distribution of 7-dehydrocholesterol was the same as in experimental rats. The preputial glands of two wild rats each contained about 0·4 mg. of 7-dehydrocholesterol per pair, or 2·5 mg./g. of wet gland. In comparison the intestines of normally nourished guinea pigs, according to Glover, Glover & Morton (1952), contain 0·1–0·4 mg./g. of wet tissue.

It is still unknown whether the 7-dehydrocholesterol in the rat genital system has any importance in supplying provitamin D, but theoretically the preputial glands contain enough for conversion into 16 000 i.u. of vitamin D_3. We have detected 7-dehydrocholesterol in the epididymes of rachitic rats, but not in those of sexually immature rats.

Investigations on other animals have so far suggested that the presence of 7-dehydrocholesterol in the male genitals is peculiar to the rat. No selective absorption was seen in extracts of the *caput epididymis* in the human, ram, guinea pig, mouse or rabbit. The preen gland of a duck, examined because of is resemblance to the preputial gland and its supposed role in supplying provitamin D (Hou, 1928) also gave an extract without selective absorption. In a human testis carotene and lycopene were detected, and an extract of the *cauda epididymis* had bands at 278 and 284 mμ. Extracts of the *caput* and *cauda epididymis* of a bull had a band at 260 mμ.

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The Reaction of Haemoglobin with Hydrogen Peroxide. By K. Dalziel and J. R. P. O'Brien. (*Department of Biochemistry (Radcliffe Infirmary), University of Oxford*)

Haemoglobin in dithionite solution, pH 7·0–9·5, is partially changed into choleglobin by reaction with hydrogen peroxide, and spectrokinetic measurements show that an unstable intermediate is formed (Dalziel & O'Brien, 1951). Within a few seconds, the haemoglobin spectrum is completely replaced by a three-banded spectrum, $\lambda_{max.}$ 417, 545 and 580 mμ., which persists with gradually diminishing intensity, and then slowly reverts to a haemoglobin spectrum of reduced intensity with a small additional peak at 630 mμ. Some haemoglobin is lost as products other than choleglobin.

The half-period for the formation of the transient compound is independent of pigment concentration and decreases with increase of peroxide concentration; the life of the compound is prolonged by increased peroxide concentration, and shortened by increased pigment or dithionite concentration, and by catalase; the half-period for the reversion to haemoglobin is independent of pigment concentration and decreases with increase of dithionite concentration. The quantitative spectral absorption and the kinetic data distinguish the compound from oxyhaemoglobin, methaemoglobin and methaemoglobin-hydrogen peroxide.

Carboxyhaemoglobin does not react with peroxide under similar conditions. Prior saturation of the peroxide solution with coal gas also largely inhibits the reaction. But if coal gas is passed into the reaction mixture during the life of the transient compound, much more choleglobin and no less haemoglobin appear in the final products as carboxy-derivatives, suggesting that carbon monoxide inhibits the further degradation of the choleglobin. Carboxyhaemoglobin is formed more slowly than when coal gas is passed into haemoglobin solution. The transient spectrum is gradually replaced by a four-banded spectrum; stable peaks at 420, 539 and 568 mμ., attributed to carboxyhaemoglobin, are fully formed after several minutes, but the fourth peak, at 600 mμ., gradually declines whilst a stable maximum at 628 mμ. builds up, the

intensity at 614 mμ. remaining constant. This change, which takes several hours to go to completion, evidently represents the formation of carboxycholeglobin from a closely related compound.

It is suggested that the transient compound is a haemoglobin-hydrogen peroxide complex, postulated by Lemberg, Legge & Lockwood (1939) as the precursor of choleglobin. The formation of choleglobin is conceived as a stage in the progressive oxidation of the complex by peroxide, presumably by attack at a methine bridge, following the coordination of a peroxide group to the iron atom.

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The Reaction of Methaemoglobin with Hydrogen Peroxide. By K. Dalziel and J. R. P. O'Brien. (*Department of Biochemistry* (*Radcliffe Infirmary*), *University of Oxford*)

The reaction of catalase-free human methaemoglobin with hydrogen peroxide has been studied spectrokinetically, in connexion with the identification of a transient compound formed when haemoglobin reacts with peroxide in dithionite solution (Dalziel & O'Brien, 1951). At pH 6·0–8·5, the initial changes of spectral absorption were consistent with the formation of the unstable complex methaemoglobin-hydrogen peroxide, with an absorption maximum at 545 mμ. and a pronounced shoulder at 575–590 mμ. (Haurowitz, 1935; Keilin & Hartree, 1935, 1951); a Soret band was recorded, $\lambda_{max.}$ 418 mμ. The extinction coefficients ($\epsilon = 1/cd$ log I_0/I, c = m-mole haematin/litre) were $\epsilon_{545} = 10{\cdot}5$ and $\epsilon_{418} = 105$, approximately. With excess peroxide, the reaction was first order with respect to both methaemoglobin and peroxide, in accordance with the equilibrium measurements of Keilin & Hartree (1951) indicating 1 molecule of peroxide per iron atom. The specific reaction rate (m-mole/l.$^{-1}$ sec.$^{-1}$) at 20° was approximately 0·25 at pH 6·0 and 0·025 at pH 8·5.

Slow decomposition of the complex was accompanied by protohaematin destruction, to an extent dependent upon pH and peroxide concentration. At pH 6·0, the spectral absorption of denatured globin carboxyhaemochromogen derivatives prepared at intervals during the reaction showed gradual decline of the carboxyprotohaemochromogen maxima at 539 and 568 mμ., and the progressive development of a maximum at 617 mμ. With an initial methaemoglobin concentration of 0·06 mM with respect to haematin, the extent of this change increased with peroxide concentration up to 0·9 mM, and isosbestic points at 510 and 585 mμ. suggested that other oxidation products were not formed to an appreciable extent. Further increases of initial peroxide concentration resulted in general destruction of specific absorption. The concomitant changes in the spectrum of the reaction mixture were more complex, but indicated that at least one stable pigment was formed in addition to methaemoglobin. The stable products with 0·9 mM-peroxide were green-brown, $\lambda_{max.}$ 585 mμ.; reduction with dithionite after saturation with coal gas gave a residual carboxyhaemoglobin spectrum with an additional peak at 613mμ. The product is evidently not choleglobin, and appears to resemble that obtained by Keilin & Hartree (1951) in the reaction of horse methaemoglobin with peroxide at pH 4·5.

At pH 8·5, no evidence was obtained for the formation of any pigment other than methaemoglobin.

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The Metabolism of Anthranilic Acid in the Rabbit and Rat. By FRANCINE CHARCONNET-HARDING, C. E. DALGLIESH and A. NEUBERGER. (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

In investigating the metabolism of tryptophan by B_2-deficient rats (cf. succeeding abstract), large amounts of three substances with a strong purple fluorescence were found in the urines. One of these was identified as anthranilic acid and the others were suspected to be anthranilic acid derivatives. This was confirmed in the present experiments.

Anthranilic acid was fed to normal and B_2-deficient rats (100 mg./day) and normal rabbits (1 g./day) and the metabolites isolated from the urine by the method of Dalgliesh (1952). In all cases examination of the urine by paper chromatography showed essentially the same picture. In butanol-acetic acid (4 : 1 : 5 mixture of Partridge, 1946) there were three main metabolites, α (R_F, 0·56, present in largest amount) and β and γ (R_F's 0·78 and 0·91 present in comparable amounts). In addition smaller amounts of other metabolites appeared, some showing fluorescence typical of anthranilic acid whilst others showed fluorescence compatible with *N*-acylanthranilic acids. These were not examined. No appreciable amounts of hydroxyanthranilic acid derivatives appeared (cf. Bray, Lake, Neal, Thorpe & Wood, 1948).

Substance γ was identified as anthranilic acid and α as its glucuronide, which is already known to be the principal metabolite of anthranilic acid (cf. Williams, 1949) and has been identified under similar circumstances by Mason (1952).

Substance β was suspected to be *o*-aminohippuric acid. This (previously unknown) substance was therefore synthesized. Comparison with β by paper chromatography in a variety of solvents, acidic, basic and neutral, showed the two to behave identically.

Glycine conjugation has been said to be inhibited by the basic *ortho* substituent of anthranilic acid (Muenzen, Cerecedo & Sherwin, 1926; Quick, 1932). It was, however, considered possibly to occur by Bray *et al.* (1948), and the present results bring anthranilic acid into line with other *o*-substituted benzoic acids which have been shown to undergo conjugation with glycine (e.g. Bray, Clowes, Thorpe, White & Wood, 1952).

Preliminary results show that separation of the metabolites can be effected on buffered celite columns.

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Riboflavin and Tryptophan Metabolism in the Rat. By FRANCINE CHARCONNET-HARDING, C. E. DALGLIESH and A. NEUBERGER. (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

The mammalian conversion of tryptophan to nicotinic acid derivatives (cf. Dalgliesh (1951) for discussion and references) must involve hydroxylation of the benzene ring derived from tryptophan, most probably by conversion of kynurenine or a derivative to 3-hydroxykynurenine or a derivative. It has been suggested (Henderson, Weinstock & Ramasarma, 1951) that riboflavin is involved in this hydroxylation.

Tryptophan was fed to (I) normal, (II) B_2-deficient and (III) B_2- and B_6-deficient rats and the urinary metabolites of tryptophan were examined by methods already described (Dalgliesh, 1952). Both young and adult rats were used.

Urines of B_2-deficient rats contained (besides minor components not examined) (*a*) anthranilic acid, its glucuronide, and *o*-aminohippuric acid which are also found in the urines of normal rats fed anthranilic acid (cf. preceding abstract), (*b*) N^{α}-acetylkynurenine, kynurenine being noted only rarely and in small amount and (*c*) kynurenic and xanthurenic acids. The xanthurenic acid excreted far exceeded that for normal rats, levels of 10 mg./100 mg. administered L-tryptophan being observed, and on adding B_2 to the diet xanthurenic acid excretion immediately returned to normal levels. The urines of normal rats fed L-tryptophan contained negligible amounts of anthranilic acid, kynurenine, hydroxy-

kynurenine or conjugated derivatives. The conversion of tryptophan to anthranilic acid derivatives in B_2-deficient rats (also briefly reported by Mason, 1952) was more marked in the young animals.

Urines from rats deficient in B_2 and B_6 contained no anthranilic acid or its metabolites. This is to be expected as B_6 deficiency inhibits kynureninase, and, moreover, promotes accumulation of kynurenine and related compounds. Both N^{α}-acetylkynurenine and 3-hydroxy-N^{α}-acetylkynurenine appeared, together with kynurenine and sometimes hydroxykynurenine (these two to a greater extent in adult than young rats) and kynurenic and xanthurenic acids.

In all animals essentially the same picture was obtained on replacing supplementary tryptophan by kynurenine, and no conversion of administered kynurenic acid to xanthurenic acid could be detected.

Tryptophan metabolism is thus markedly affected by riboflavin. Riboflavin deficiency does not result in inhibition of hydroxylation (e.g. the high output of xanthurenic acid) but appears to cause diversion, by means not yet clear, of the metabolic pathways to end products of little value.

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Some Properties of Vitamin B_{12}-like Factors from Calf Faeces. 3. Further Biological Properties and Interrelationships. By J. E. Ford, S. K. Kon and J. W. G. Porter. (*National Institute for Research in Dairying, University of Reading*)

We have shown (Ford, Kon & Porter, 1951; Ford & Porter, 1952) that extracts of calf faeces contain four substances having vitamin B_{12} activity for *Escherichia coli*: fraction *A* (now provisionally called cyano-ω-cobalamin), fractions *B* and *C*, and vitamin B_{12} itself.

When *Esch. coli* was grown in a vitamin B_{12}-free basal medium supplemented with fraction *B* (inactive for *Lactobacillus leichmannii*) extracts of the harvested cells contained only fraction *C*, together occasionally with cyano-ω-cobalamin, both markedly active for *Lb. leichmannii*.

Cyano-ω-cobalamin and vitamin B_{12} were recovered largely unchanged after similar passage through *Esch. coli*, although traces of fraction *C* were occasionally detected.

Experiments with chicks depleted of vitamin B_{12} showed that cyano-ω-cobalamin, fraction *B** and vitamin B_{12} injected intramuscularly were partly retained in the livers. Bioautographs of extracts of the livers showed no evidence of interconvertibility of the different substances.

In *in vitro* experiments cyano-ω-cobalamin, fraction *B* or vitamin B_{12} was incubated at 37° with minced livers of depleted chicks in the presence of 0·36 % glucose in Krebs bicarbonate saline in equilibrium with 5 % CO_2 and 95 % O_2. Here again, the different substances appeared unchanged in the extracts after incubation.

Groups of depleted chicks were given cyano-ω-cobalamin (2 or 20 μg./bird), fraction *B* (100 μg.†/bird) or vitamin B_{12} (2 μg./bird) mixed in the basal diet. Vitamin B_{12} was well absorbed and appeared in a concentration of up to 0·4 μg./liver. Cyano-ω-cobalamin was also taken up and appeared as such in the livers, though in much smaller amounts than vitamin B_{12}, even when given at the higher level. Fraction *B* was taken up little, if at all, since it could not be detected in the livers. In some birds, however, the feeding of fraction *B* was accompanied by the appearance of traces of vitamin B_{12} in the livers.

The behaviour of cyano-ω-cobalamin and fractions *B* and *C* suggests that they may be involved in the metabolism of the intestinal flora rather than in that of the host.

We are indebted to our colleague Dr M. E. Coates for the chicks.

* The larger quantities of fraction *B* used in the experiments with chicks were, as before, kindly supplied by Dr E. Lester Smith (cf. Coates, Ford, Harrison, Kon & Porter, 1952).

† As judged by *Esch. coli* assay.

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The Metabolism of *n*-Valeric Acid and Some Branched Chain Acids by Sheep Tissues *in vitro*

By E. F. ANNISON and R. J. PENNINGTON. (*Rowett Research Institute, Bucksburn, Aberdeenshire*)

n-Valeric acid and branched-chain fatty acids together comprise a few per cent of the total volatile acid normally present in the sheep rumen and probably result, at least in part, from deamination of amino-acids by micro-organisms in the rumen (Shazly, 1952). They are probably absorbed from the rumen, as are acetic, propionic and butyric acids (Elsden & Phillipson, 1948), the main fatty acids found in the rumen.

It has recently been shown (Pennington, 1952) that the last-mentioned acids are metabolized by the epithelium of the rumen itself and by other sheep tissues. In the present experiments the fate of *n*-valeric, *iso*butyric, *iso*valeric and racemic α-methylbutyric acids incubated *in vitro* with sheep tissues has been studied. All were found to be metabolized by rumen epithelium; only *n*- and *iso*valeric acids caused an increase in ketone body production over the controls. Generally similar results were obtained with liver slices. Kidney slices metabolized the acids, but with negligible ketone body production with all acids.

*iso*Valeric acid markedly depressed the oxygen uptake of rumen epithelium; Q_{O_2} was increased by the other acids. The mechanism of the respiratory depression is not known; it has been observed also with propionic acid (Pennington, unpublished results).

The uptake of *iso*valeric acid by rumen and liver tissue was much greater in the presence of CO_2 (at the same pH). This observation is in line with the suggestion made by Coon (1950), on the basis of tracer studies, that a CO_2 fixation reaction is involved in the metabolism of *iso*valeric acid by liver tissue.

To investigate whether any of the acids were broken down into lower acids by the tissues, the filtrates, after incubation, were analysed using the gas-liquid partition chromatographic method of James & Martin (1952). Definite evidence of such breakdown was obtained; for example, *n*-valeric acid gave rise to appreciable quantities of acetic and propionic acids.

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Attempts to Render Growth Hormone Non-diabetogenic. By E. REID (Beit Memorial Research Fellow). (*School of Biochemistry, University of Cambridge*)

The well established finding that diabetes can be induced in cats or dogs by highly purified growth-hormone (GH) preparations, obtained from cattle pituitary glands, does not exclude the possibility that this activity depends on the presence in the preparations of some contaminant. Some evidence against this possibility was obtained by Reid (1952), who subjected such preparations to treatments likely to cause partial inactivation, and in no case found a significant alteration in the 'D/G ratio'—the ratio of diabetogenic activity (assayed in cats) to growth-promoting activity (assayed in rats). In an attempt to obtain further evidence, assays have now been performed on GH preparations treated with thioglycollate, alkali, carboxypeptidase, or oxidized cellulose.

Considerable loss of growth-promoting activity occurred when purified GH preparations were treated with thioglycollate at pH 7·8, or were maintained for 2 days in the cold at pH 13. Growth-promoting activity did not, however, diminish when GH was incubated with carboxypeptidase (Armour) in the presence of di*iso*propyl fluorophosphonate, which served to inhibit the action of any chymotrypsin present in the enzyme preparation (cf. Sanger & Thompson, 1952). Liberation of lysine, serine, alanine, leucine and phenylalanine was demonstrated by partition chromatography. Condliffe & Li (1952) have reported similar findings in experiments with this enzyme.

None of these treatments markedly altered the D/G ratio. The assay data for certain of the treated preparations were suggestive of a rise in the ratio, but in no case were such apparent rises shown to be significant.

Oxidized cellulose has a marked affinity for ACTH but not for GH, and has been used in purifying GH obtained from pig pituitary glands (Raben & Westermeyer, 1951). Pig GH obtained thus, and dissolved at pH 3·5, was tested in dogs by

Raben & Westermeyer (1952), and found to have no diabetogenic activity at doses much higher than the effective dose of beef GH.

Tests now performed on GH preparations treated with oxidized cellulose have not, up to the present, indicated any significant lowering of the D/G ratio. The preparations under test include purified GH (ox), partly purified GH (ox) obtained by the procedure of Raben & Westermeyer (1951), and purified GH (pig) prepared by these authors and kindly provided by them through the courtesy of Prof. E. B. Astwood. This pig preparation, dissolved at pH 10 since it was insoluble at mildly alkaline pH values, has manifested diabetogenic activity in preliminary tests, and is now being quantitatively assayed.

The present experiments have so far given no conclusive evidence against the view previously expressed (Reid, 1952)—that diabetogenic and growth-promoting activity probably depend on the same structures in the same molecule.

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The Direct Determination of Nitrogen in Protein Fractions separated by Electrophoresis in Filter Paper. By A. B. Anderson. (*Biochemical Laboratory, North Middlesex Hospital, London, N.* 18)

The several methods of staining protein fractions after electrophoresis in filter paper, which have been advocated, all have the disadvantage that the dye absorption power of the different fractions varies. The present communication describes a method for the determination of the protein nitrogen directly in the paper. Electrophoresis of serum on strips of Whatman no. 31 paper was carried out in a tank as described by Flynn & de Mayo (1951) using a borate buffer pH 8·7 (Sørensen). At the end of the run the papers were dried at 110° and cut transversely into a series of strips 5 mm. wide. These small strips were individually digested with sulphuric acid selenium dioxide mixture and the nitrogen in the digests determined by nesslerization. Blank determinations on protein free portions of the papers gave a reasonably constant figure.

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Enzymic Oxidation of 5-Hydroxytryptamine in Mammalian and Cephalopod Tissue. By H. Blaschko. (*Department of Pharmacology, University of Oxford*)

Two substances isolated from animal sources are said to be identical with 5-hydroxytryptamine; one is the vasoconstrictor substance serotonin (Rapport, 1949), the other is enteramine (Erspamer & Asero, 1952).

A sample of serotonin creatinine sulphate and one of enteramine picrate have been examined as possible substrates of amine oxidase. The two samples were incubated with extracts from guinea pig tissue and from posterior salivary glands of *Octopus vulgaris*. The rates of oxidation were compared with those of tyramine and tryptamine; it was found that both samples were oxidized by the two tissue preparations at a rate similar to the rate of oxidation of tyramine and of tryptamine. Like tryptamine, 5-hydroxytryptamine forms a dark pigment in the course of the oxidation.

The interest in these observations lies in the high rate of oxidation of 5-hydroxytryptamine; if the amine is proved to be a normal tissue constituent, it may be an important substrate of amine oxidase in the living tissue.

The author is grateful to Prof. J. H. Gaddum and to Dr W. Feldberg for the two samples used in these experiments.

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The Influence of Growth Hormone on the Protein Composition of Rat Muscle. By B. J. HUME (introduced by F. G. YOUNG). (*Biochemical Laboratory, University of Cambridge*)

Bigland & Jehring (1951) showed that the quadriceps muscle from a growth hormone-treated rat was not capable of producing any greater tension than that from a control. Compared gram for gram the treated muscle was actually weaker than the control, suggesting that the extra material laid down under the influence of growth hormone is of a non-contractile nature. A fractionation of the muscle protein has therefore been carried out in order to investigate this problem further.

By extracting with 0·1 M-KCl at 0° followed by 0·1 N-NaOH the muscle protein was divided into three fractions, the sarcoplasmic protein, the myofibrillar protein, and collagen and elastin. Although this procedure does not give a completely clear-cut separation any differences in the gross composition of the muscle protein should be apparent. The results showed no such differences, nor any change in the water content, indicating that the relative proportions of the components of the muscle remained unchanged after growth hormone treatment. Treatment with growth hormone plus testosterone propionate caused a larger increase in weight than with growth hormone alone, but as before the relative composition of the muscle remained unchanged.

In addition, determinations of the 'myofibrillar adenosinetriphosphatase (ATP-ase)', the 'granular ATP-ase' and the 'total ATP-ase' were carried out by a slight modification of the method of Perry (1951, 1952), using homogenates from the quadriceps muscle of growth hormone treated and control rats. A significant decrease was observed in the ATP-ase activity associated with the myofibrils, suggesting that there was a decrease in the relative proportion of myofibrillar protein present. This is in agreement with the findings of Bigland & Jehring that the protein laid down under the influence of growth hormone is not identical with normal muscle protein. The absence of any change in the gross composition of the muscle as determined by the extraction procedure used, is not incompatible with this finding. The part estimated as myosin might be a precursor of myosin or some inert protein of the same nature as myosin but laid down in an unorganized manner. It would then have no ATP-ase activity nor add to the contractile strength of the muscle. Other possible explanations of the findings are also considered.

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Blood Adrenaline Levels During Insulin Hypoglycaemia. By H. WEIL-MALHERBE. (*Research Department, Runwell Hospital, Wickford, Essex*)

Most existing methods for the estimation of adrenaline in blood are unsatisfactory owing to their lack of specificity or sensitivity or both. A method has recently been described (Weil-Malherbe & Bone, 1952) with which, as we believe, fairly reliable values for the sum total of adrenergic amines may be obtained, though it is not possible at present to differentiate between adrenaline and noradrenaline. The term 'blood adrenaline' is here used to signify the total of adrenergic amines estimated by the method without implications as to the identity of the constituents or the composition of the mixture.

The method has been applied to the investigation of adrenaline levels in human venous blood during insulin hypoglycaemia. Many well established observations indicate a state of increased adrenergic discharge during hypoglycaemia; nevertheless, it was found that insulin produces a pronounced fall of the adrenaline concentration in venous blood. The effect is practically instantaneous: after an intravenous injection of insulin the adrenaline concentration curve usually reaches its minimum within 5 min., while the blood sugar may still be within normoglycaemic limits. Thereafter the blood adrenaline level tends to show a gradual rise depending on the dose injected and the response of the subject. If the dose is small (0·1 unit/kg.) and the subject is not abnormally sensitive, the normal adrenaline concentration may be restored after about 20 min., while the blood sugar is still decreasing. Where the response or the dose is greater, the rise of the blood adrenaline is more protracted and the return to the starting level delayed. In any case, a spontaneous upward movement of the blood sugar curve is always preceded by a cor-

responding movement of the blood adrenaline curve. If coma doses of insulin are administered, the adrenaline level remains depressed until the coma is terminated by suitable intervention. In many cases this may be done successfully by the intravenous injection of certain amino-acids (glutamic acid, arginine) or by electric stimulation across the skull. These procedures often lead to a sustained rise of the blood adrenaline up to the starting level or above, followed by a return of consciousness. After the administration of glucose by stomach tube the venous adrenaline level returns to normal within 10–15 min. and often continues to rise for some time afterwards.

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Vitamin A and the Resistance of Rats to Protein Deficiency. By T. Moore, I. M. Sharman and R. J. Ward. (*Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council*)

It has been shown repeatedly that the ability of rats to survive upon diets low in protein is influenced by the intake of vitamin E. In view of the known synergism between vitamins E and A it seemed desirable to find out whether the adequacy of the vitamin A allowance might also affect the resistance to protein deficiency. Eight young male piebald rats were first kept for about 3 months on a diet deficient in vitamin A, but adequate in vitamin E and protein, which was supplemented after the first few weeks with small doses of vitamin A inadequate to allow the deposition of liver reserves. They were then transferred, at body weights of 219–257 g., to a diet containing 10 % of dried yeast as the only source of protein. Four of the animals were dosed with 5000 i.u. of vitamin A acetate daily for 3 days, and subsequently were given 1000 i.u. weekly. With these liberal doses they all increased slightly in weight, and in spite of subsequent declines their mean weight after 10 weeks of dosing was 9 g. above that which had been observed when dosing was started. The other four rats were dosed with only 28 i.u. of vitamin A acetate weekly, which was an allowance calculated to have allowed moderately rapid growth if the basal diet had contained an adequate allowance of protein. All these animals declined in weight. One died after 8 weeks of protein restriction, and after 10 weeks the mean weight of the remaining three rats was 30 g. below the weight at the time when dosing was started. When the same dosage of vitamin A was supplemented by the restoration of an adequate allowance of casein, however, the rats commenced to grow rapidly, with a mean gain of 24 g. in one week. Under the particular conditions of this preliminary experiment, therefore, a liberal allowance of vitamin A enabled the animals at least partially to resist the ill effects of a severe deficiency of protein.

Precursors of Porphyrin in the Urine in Idiopathic Porphyria. By F. K. Herbert. (*Section of Chemical Pathology, Department of Pathology, King's College, Newcastle-upon-Tyne*)

Waldenström & Vahlquist (1939) described the formation of porphobilin and ether-insoluble porphyrins on heating porphobilinogen in acid solution and this was confirmed by Prunty (1945). The increased yield of porphyrin obtained on heating the crude acidified urine, in cases of idiopathic porphyria, has usually been attributed to the formation of porphyrin from porphobilinogen. The recognition of a different type of precursor of coproporphyrin soluble in organic solvents and converted to the porphyrin on oxidation (Raine, 1950; Watson, de Mello, Schwartz, Hawkinson & Bossenmaier, 1951; Eriksen, 1951) suggested the search for an analogous precursor of 'uroporphyrin'. Watson *et al.* (1951) described such a precursor in idiopathic porphyria, separated it from porphobilinogen, and stated that on heating at pH 4·0 porphobilinogen yielded only porphobilin, whereas the new precursor yielded porphyrin.

In the present work, a 'uroporphyrin' precursor, soluble in ethyl acetate and converted to porphyrin on oxidation, has been found in three cases of idiopathic porphyria (in freshly passed urine protected from light). It has been separated from porphobilinogen. Porphobilinogen, as prepared in purified solution by chromatography on alumina, yields both porphobilin and porphyrin on heating in acid solution. The rates of formation of porphyrin and porphobilin, and destruction of porphobilinogen, have been followed in buffered and unbuffered

solutions. In 0·01M-citrate at pH 5·2 the yield of porphobilin is high and porphyrin low. Large amounts of buffer salts suppress porphyrin formation. Maximum porphyrin yields occur in 0·05–0·20N-HCl without buffer salts.

Porphobilinogen solutions in concentrations up to 20 Vahlquist units show no characteristic ultraviolet absorption spectrum. On heating in HCl, the bands of porphyrin and porphobilin develop. In citrate at pH 5·2 the changes on heating are complex and include the transient formation of a weak band at 350–370 mμ. as reported by Brockman & Gray (1951).

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DEMONSTRATIONS

The 'Chromatocoil'—a space-saving device for Paper Chromatograms. By V. SCHWARZ.
(*Research Division, Allen and Hanburys Ltd., Ware, Herts*)

An apparatus has been designed which is small enough to be accommodated in an incubator or refrigerator and thus enables paper chromatograms to be run at any desired temperature.

A wide-mouthed glass-stoppered jar (4·5 cm. diam., 10–11 cm. high) is fitted with a short side arm, also carrying a glass stopper. The jar has two indentations on opposite sides to support a glass rod at a level just below the stopper. Two strips of duralumin, about 5·3 × 1·3 cm., are furnished with 1 cm. slots in such a way as to form four or five prongs (1·5 mm. wide) at both ends of the strips. The two pieces are then fitted together at right angles to each other, forming a cross-shaped holder round which the chromatogram strip can be wound in coil form. The holder carrying the paper strip is suspended from the glass rod and fitted into the jar. A small beaker (5 ml.) contains the stationary phase, while the jar itself holds the moving phase. After equilibration more solvent is added through the side arm until the level just reaches the end of the paper strip projecting towards the bottom. Chromatographic development is thus started and continues until the solvent front reaches the end of the strip when it stops automatically.

Experience has shown that neither the coiling of the strip nor its contact with the prongs of the holder has any untoward effect. The advantages of the apparatus, apart from the great saving in space and hence its ready accommodation in an incubator, are: the short time necessary for equilibration (1–2 hr.); the small volumes of solvents required; the ease with which it can be made in the laboratory.

The use of the apparatus for the chromatography of cortical steroids by the method of Bush (1952) and their location by the arsenomolybdate technique (Schwarz, 1952*a*, *b*) are demonstrated.

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Cytochrome components in Chloroplasts. By H. E. DAVENPORT.

Glycosides and a Glycosidase in the Madder Plant. By A. R. TRIM and R. HILL.