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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **321st Meeting** *of the Biochemical Society was held in the Department of Biochemistry, The School of Medicine, Leeds* 2, *on Friday, September* 18*th*, 1953, *at* 11 *a.m., when the following papers were read:*

COMMUNICATIONS

Oxidative Phosphorylation in Insect Sarcosomes.

By S. E. LEWIS and E. C. SLATER. (*Molteno Institute, University of Cambridge*)

Sarcosomes in the flight muscles of insects resemble mammalian heart-muscle sarcosomes in their morphology (see Cleland & Slater (1953) for review), their high concentration of cytochrome (Keilin, 1925; Levenbook, 1952) and their high respiratory activity (Watanabe & Williams, 1951). However, attempts to link this respiratory activity with the synthesis of adenosine triphosphate (ATP) have up to now been unsuccessful (see Sacktor, 1953).

Using the procedure previously described (Slater, 1953), oxidative phosphorylation has now been demonstrated during the oxidation of α-ketoglutarate by sarcosomes obtained from the flight muscles of the blowfly *Calliphora erythrocephala* (2–6 days old). After removing the head and abdomen, the flies were briefly ground without sand in a mortar with 0·20M-sucrose, 0·002M-ethylenediaminetetraacetic acid (Versene) and the sarcosomes separated by differential centrifugation. In a typical experiment with 2-day-old flies, sarcosomes containing 1·2 mg. protein consumed 2·01 μatoms oxygen and 1·08 μmoles α-ketoglutarate, and 2·07 μmoles of phosphorus were esterified (P : O = 1·03). Under the same conditions, with the same preparation, the P : O ratio with succinate as substrate was 0·14. The P : O ratio for the oxidation of α-ketoglutarate to succinate is, therefore, $\{2{\cdot}07 - 0{\cdot}14\ (2{\cdot}01 - 1{\cdot}08)\}/1{\cdot}08 = 1{\cdot}80$. This is lower than the value of 3 obtained for heart-muscle sarcosomes (Slater & Holton, 1953). The low ratio is not due to high ATP-ase activity, since sufficient hexokinase was added to compete with the ATP-ase completely. Neither is it due to phosphomonoesterase activity, which is very small in these sarcosomes. It cannot yet be stated whether the low ratio is a property of insect sarcosomes *in vivo*, or is due to losses of phosphorylating enzymes during the isolation procedure.

S. E. L. is on leave from D.S.I.R. Pest Infestation Laboratory, London Rd., Slough. E. C. S. is working on behalf of the Agricultural Research Council.

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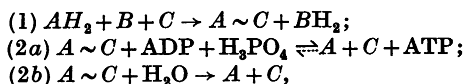
The Effect of Dinitrophenol on Insect Sarcosomes.

By E. C. SLATER and S. E. LEWIS. (*Molteno Institute, University of Cambridge*)

The stimulation of the respiration of mitochondria by the addition of either dinitrophenol or adenosinetriphosphate (ATP)-utilizing systems such as hexokinase and glucose is now well known. However, with liver mitochondria (Lardy & Wellman, 1952) or heart-muscle sarcosomes (Slater, unpublished) these two effects are not additive, i.e. the addition of dinitrophenol to mitochondria already supplied with excess hexokinase and glucose has little effect on the respiration. In the case of insect sarcosomes, however, the addition of 10^{-4} M-dinitrophenol increased the rate of oxidation of α-ketoglutarate 3·2-fold, even in the presence of excess hexokinase and glucose. This concentration of dinitrophenol decreased the P : O ratio from 1·03 to 0·62 (P : α-ketoglutarate, 0·83), but the increase of respiration more than compensated for the lower ratio, with the unusual result that the rate of synthesis of esterified phosphorus was increased by 36%. This is a consequence of three factors: (1) the substrate-linked phosphorylation with α-ketoglutarate is little affected by dinitrophenol (Judah, 1951; Hunter, 1951); (2) the dinitrophenol-sensitive respiratory chain phosphorylation contributes a relatively small

proportion of the total phosphorylation in the insect-muscle sarcosomes; (3) the rate of respiration is limited by some process which is affected by dinitrophenol.

These findings are understandable on the basis of the following description of respiratory chain phosphorylation (cf. Hunter, 1951; Lardy & Wellman, 1953):



$$(1)\ AH_2 + B + C \rightarrow A \sim C + BH_2;$$
$$(2a)\ A \sim C + ADP + H_3PO_4 \rightleftharpoons A + C + ATP;$$
$$(2b)\ A \sim C + H_2O \rightarrow A + C,$$

where A and B are adjacent hydrogen-carriers in the respiratory chain and C is a substance or grouping essential for their interraction. The rate of respiration is governed by the regeneration of A by reactions (2a) and (2b). Reaction (2a) is not rate-limiting in heart sarcosomes which give a high P : O ratio, but is slow in the poorly phosphorylating insect sarcosomes. Reaction (2b) is normally relatively slow, but is accelerated by dinitrophenol. The reverse reaction (2a) is not operative in our experiments, because ATP is rapidly removed by the hexokinase-glucose.

The finding that the rate of synthesis of ATP can be increased by the addition of a typical uncoupling agent, even when the ATP is being rapidly utilized, is some support of Lardy's (1952) theory of the mode of action of certain hormones.

E. C. S. is working on behalf of the Agricultural Research Council. S. E. L. is on leave from D.S.I.R. Pest Infestation Laboratory, London Road, Slough.

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A Study of the Complexes of Some Simple Peptides with Zinc and Cadmium Ions in Aqueous Solution.

By D. J. Perkins. (Introduced by N. H. Martin.) (*Department of Chemical Pathology, St George's Hospital Medical School, London, S.W.* 1)

The zinc and cadmium complexes of a number of glycyl dipeptides and some other simple peptides have been examined by a potentiometric method. The effect of peptide structure on the stability constants of the metal complexes has been studied. The presence of peptide bonds was found to decrease these constants when compared with those of the constituent amino acids. The relative effects of the structure of each amino acid in a series of glycyl peptides was found to be similar to those for the free amino acids.

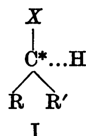
The Molecular Rotations of Triterpenoid Alcohols and their Derivatives.

By W. Klyne (*Postgraduate Medical School, London, W.* 12) and W. M. Stokes (*Providence College, Providence, R.I., U.S.A.*)

Stokes & Bergmann (1952) discussed the rotation contributions of hydroxyl and related groups in steroids, and tried to apply to these compounds the empirical rules of Marker (1936) regarding acyclic compounds. Mills (1953) showed by reference to monoterpenoids that the application of Marker's rules to cyclic compounds is unjustified. The work of Mills (1952, 1953) on optical rotations and of Dauben, Dickel, Jeger & Prelog (1953) on asymmetric synthesis has shown that the steroid and glyceraldehyde conventions for formulae are in agreement. Klyne (1952) had previously correlated the triterpenoids with the steroids.

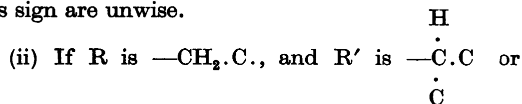
The method of rotational analysis used by Stokes & Bergmann (1952) can now be applied generally to cyclic alcohols in the light of the work of Mills and others cited above. The principles are as follows. An asymmetric centre C* in a *cyclo*hexane ring system may be represented in perspective as in (I), where X is a substituent and R, R′ are the ring-

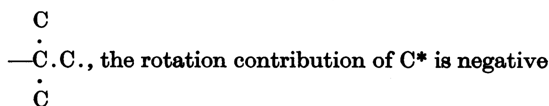
members adjacent to C* together with their substituents; the hydrogen atom (H) is on that side of C* farthest from the observer.



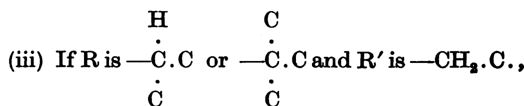
If X is a hydroxyl, acetoxyl or benzoyloxy group, the magnitude and sign of the rotation contribution of C* is dependent on the degree of alkyl substitution in R and R′.

(i) If R and R′ are both —CH_2.C., the rotation contribution of C* is small and attempts to predict its sign are unwise.





(and often large).



the rotation contribution of C* is positive (and often large). The contributions of acetoxyl and benzoyloxy groups are generally larger than those of the corresponding hydroxyl groups. These rules probably apply also to *cyclo*pentane rings.

The results for triterpenoids are with few exceptions consistent within themselves and with other evidence regarding the stereochemistry of the triterpenoids.

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Synthesis and Oxidation of Citric Acid in Mammary Gland Homogenates. By C. TERNER. (*National Institute for Research in Dairying, University of Reading*)

Evidence presented previously (Terner, 1951) supports the view that the citric acid cycle is operating in the mammary gland. Moore & Nelson (1952), using a cylophorase preparation of lactating guinea pig mammary gland, found that added citrate was oxidized only slowly if at all and concluded that 'not only does mammary tissue not oxidize citrate, but it contains an inhibitor to this oxidative step of the tricarboxylic acid cycle'.

Since citric acid is an intermediate of the Krebs cycle, the inability of the mammary gland to oxidize citrate at adequate rates would rule out the cycle as a major metabolic pathway in this tissue.

In the present work whole homogenates of lactating mammary gland of the guinea pig were found capable of synthesizing citrate from pyruvate or acetate in the presence of fumarate and of oxidizing added citrate at rapid rates. Pyruvate was removed more rapidly than added citrate and the amount of citrate accumulating during the metabolism of pyruvate was approximately equivalent to the difference between the amounts of pyruvate and of added citrate disappearing. In the presence of fluoroacetate (see Liebecq & Peters, 1949), fumarate and pyruvate, when present in equivalent amounts, were almost quantitatively converted to citrate, while either fumarate or pyruvate alone gave rise to only small amounts of citrate.

The homogenates had a considerable endogenous respiration which was not greatly increased by the addition of pyruvate, acetate or citrate, although these substances could be shown by analytical methods to be actively metabolized. *p*-Nitrophenol (*p*NP) which had little effect on the endogenous respiration or on the respiration in the presence of fumarate alone, caused a large increase of respiration in the presence of fumarate plus pyruvate or acetate, and of citrate or α-ketoglutarate, without correspondingly accelerating the rate of disappearance of these substrates. Thus, *p*NP increased the ratio O_2 consumed/substrate oxidized to a value approaching the theoretical ratio for the complete oxidation of the substrate. According to these findings various substrates appear to be oxidized incompletely in mammary homogenates. The observation of incomplete oxidation made by analytical

and respiratory measurements aided by the use of nitrophenols has been taken as evidence suggestive of the utilization of the substrates in synthetic reactions (Terner, 1951; Melrose & Terner, 1953). In the presence of fluoroacetate the amounts of oxygen taken up, pyruvate disappearing and citrate accumulating were approximately equivalent and were increased by *p*NP to the same extent.

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The Enzyme Complement of Bean Root Cells. By EDITH ROBINSON. (*Department of Botany, Leeds University*)

The bean root is suitable experimental material for the study of metabolic changes which occur during the growth of the plant cell. Serial sections cut from the apex of the root towards the base contain cells in progressively more advanced stages of development ranging from the meristematic to the mature state. Quantitative measurements made with such sections can be reduced to a 'per cell' basis by dividing the section values by the number of cells each contains. Estimations of growth, protein content, invertase, dipeptidase, phosphatase, glycine oxidase and proteolytic enzyme activity have been made. The activity of all enzymes changes during the growth of the cell. Invertase, phosphatase, dipeptidase and glycine oxidase show maximum activity, and protein content is highest when the cell reaches its maximum length. All these quantities decrease after growth ceases. Activity of proteolytic enzymes continues to increase in cells which finished growth. It is tentatively suggested that the level of proteolytic enzymes may determine the decrease in activity of the other enzymes studied.

The Separation of α-Keto Acids in Blood and Urine by Paper Chromatography. By M. J. H. SMITH and K. W. TAYLOR. (*Department of Chemical Pathology, King's College Hospital Medical School, Denmark Hill, London, S.E.* 5)

1:2-Diamino-4-nitrobenzene (DANB) reacts with α-keto acids to yield stable derivatives which may be separated by paper chromatography (Hockenhull & Floodgate, 1952; Hockenhull, Hunter & Herbert, 1953). A satisfactory separation of the DANB derivatives of pyruvic, α-ketoglutaric, oxaloacetic, phenylpyruvic and α-ketobutyric acids has been achieved using Whatman no. 4 paper and a solvent mixture consisting of ethanol, amyl alcohol and 0·880 ammonia solution. The derivatives form yellow spots which show a deep yellow fluorescence in ultraviolet light. The R_F values show slight variation from day to day, due probably to minor changes in the solvent mixture and environmental temperature. The ranges of R_F values found for the DANB derivatives are 0·10–0·15 for oxaloacetic acid, 0·24–0·35 for α-ketoglutaric acid, 0·55–0·66 for pyruvic acid, 0·68–0·74 for α-ketobutyric acid and 0·78–0·85 for phenylpyruvic acid.

DANB has been used for the detection of α-keto acids in blood and urine. The reagent in acid solution is added to the urine or to the filtrate from blood which has been deproteinized by metaphosphoric acid. The derivatives are extracted with ethyl acetate and then removed from the organic solvent with caustic soda solution. After acidification of the caustic soda solution the derivatives are re-extracted with ethyl acetate which is evaporated to dryness and the residue dissolved in ethanol, the ethanolic solution being applied to the paper for chromatography.

Pyruvic and α-ketoglutaric acids were always found in blood and urine samples from normal subjects and diabetic patients, but oxaloacetic acid was never detected (cf. El Hawary & Thompson, 1953). A spot with an R_F value of between 0·86 and 0·93 was seen in about half of the chromatograms, but has not yet been identified. Phenylpyruvic acid was found in the urine of two cases of phenylpyruvic oligophrenia. Pyruvic and α-ketoglutaric acids only were detected in the urine of normal rats.

The quantitative estimation of the α-keto acids in blood and urine is being studied by elution of the spots with ethanol and measurement of the optical densities of the resulting solutions at 280 mμ.

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Effect of Inhibitors on the Metabolism of *Microsporum*. By A. J. E. Barlow, F. W. Chattaway and C. C. Thompson. (*Department of Dermatology, Huddersfield Royal Infirmary, Department of Biochemistry, School of Medicine, Leeds*)

Endogenous respiration of *Microsporon canis* has been studied by Melton (1951) who examined the effect of various fungicidal substances on the respiration of shake cultures, and Bentley (1953) has determined the Q_{O_2} of mycelial mats. Using mycelial mats of *M. canis* and *M. audouini* grown on Sabouraud's broth at room temperature for 14–20 days it has been shown that fluoride (0·1 M), arsenite (0·01 M), iodoacetate (0·017 M) and cyanide (0·001 M) are powerful inhibitors of endogenous respiration. Fluoroacetate (0·036 M) and malonate (0·03 M) had only a slight inhibitory effect. The mycelial mats showed a respiratory quotient of 1 and Q_{O_2} (N) values were in the range 100–200.

Preformed mycelial mats of these organisms floated on to replacement medium containing phosphate buffer (0·066 M, pH 7) and potassium arsenite (0·01 M) and incubated at room temperature for 6 days lead to the accumulation in the medium of pyruvic and α-ketoglutaric acids and a third unidentified keto acid. The first two of these acids have been identified by chromatography, ultraviolet absorption spectrum and m.p. of their 2:4-dinitrophenylhydrazones and the pyruvate derivative by analysis also. The corresponding derivative of the third acid, obtained in low yield, has $\lambda_{max.}$ 355 mμ and $\lambda_{min.}$ 300 mμ in ethyl acetate. The yield of total keto acids is considerably increased by the addition of glucose (0·1 M) to the medium but not by galactose, sucrose or succinic acid and only slightly by mannose. The addition of fluoride (0·15 M) or iodoacetate (0·02 M) to the arsenite-glucose medium reduces the yield of total keto acid by 90%. Total keto-acid production in the presence of glucose (0·1 M) is of the order of 150 μg./ml. and is not greatly altered by pH, between pH 5–7; arsenite is essential for accumulation of keto acids to occur at all pH values. These findings are similar to those of Hockenhull, Wilkin & Winder (1951) who found accumulation of pyruvate and α-ketoglutarate in high yield in cultures of *Penicillium chrysogenum*, arsenite being essential at pH 7 but not at pH 5·4.

No significant differences have been found in the behaviour of *Microsporon canis* and *M. audouini* and the results provide preliminary evidence as to possible mechanisms for carbohydrate metabolism in these fungi.

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The Site of Conversion of Carotene Injected into Rats. By J. G. Bieri and C. J. Pollard (introduced by S. K. Kon). (*Department of Biochemistry and Nutrition, The University of Texas, Galveston, U.S.A.*)

When carotene solubilized in water with Tween 40 (Bieri, 1951) is injected intravenously into vitamin A-deficient rats containing only traces of vitamin A in the serum and organs high levels of vitamin A in the serum result within a few hours (Bieri & Pollard, 1953). In order to obtain information as to the possible site of conversion of the injected carotene, a series of studies was carried out with rats subjected to various surgical procedures before the injection of carotene. Deficient rats weighing 100–140 g. were operated upon under ether anaesthesia and immediately given 75–100 μg. β-carotene in aqueous solution through the tail vein. After 3–8 hr. the animals were killed and vitamin A and carotene determined in the serum, liver and kidneys.

Control rats, either intact or subjected to mock operations, produced serum vitamin A levels of 57–157 μg./100 ml. after injecting carotene. From

2·7 to 5·8 μg. vitamin A per rat were in the combined livers and kidneys. When the bile duct was ligated and severed, the vitamin A resulting from injected carotene was 93–102 μg./100 ml. serum, and 3·3–3·9 μg. in the liver and kidneys. Thus, the carotene was not transported by the bile to the intestine for conversion.

When the small intestine of rats was removed before administration of carotene, the conversion was not impaired. Vitamin A in the serum ranged from 24 to 107 μg./100 ml., and from 1·0 to 5·1 μg. were in the liver kidneys. The small intestine thus does not play a significant part in converting injected carotene.

Removal of the kidneys before carotene injection did not alter the amount of vitamin A formed, (72–161 μg./100 ml. serum, and 1·5–2·3 μg. per liver). When 60–75 % of the liver was removed and the animals injected, appreciable vitamin A was still found. The sera contained 20–76 μg./100 ml., and the remaining liver with the kidneys had 1·2–4·6 μg.

The variations in survival time and in the dosage of carotene in the different experiments, as well as the individual variation among rats, do not make it possible to state that the removal of any organ affected the conversion of carotene to vitamin A more than did the removal of another organ. It is evident that with either the small intestine or more than one-half of the liver removed, the formation of vitamin A from injected carotene is not appreciably reduced.

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Tryptophan-Tryptophanase Reaction. Consideration of the Nature of the Enzyme-Coenzyme-Substrate Complex.

By H. Gooder and Frank C. Happold. (*Department of Biochemistry, The University, Leeds*)

A study of the effect of certain structural analogues of tryptophan upon the tryptophan-tryptophanase reaction shows that:

(*a*) Indole and indolyl acids inhibited the reaction strongly while slight inhibition was given by α-amino-β-3-indenyl-propionic acid when preincubated with the enzyme.

(*b*) Indene, D-tryptophan, indolyl ethylamine and *N*-methyl tryptophan failed to inhibit the reaction.

The enzyme system did not produce indole with any indole containing substrate other than L-tryptophan nor was ammonia or pyruvic acid produced from α-amino-β-3-indenyl-propionic acid.

The relationship of these results to possible mechanisms of the enzyme reaction is discussed.

It is suggested that combination between L-tryptophan and the enzyme surface occurs through the ring nitrogen atom as also through the carboxyl group of the substrate. The L-tryptophan may combine with the coenzyme, pyridoxal phosphate, through the amino group of the former and the free carboxyl of the latter; the coenzyme being attached to the apo-enzyme by the phosphate group of the pyridoxal phosphate. Such a complex would provide a highly resonating network in which electron transfers could take place, and under correct energy conditions these could rupture the C_3–C_β bond in the tryptophan molecule. No intermediate would be demonstrated under these conditions since the other entity produced (in addition to indole) could be an α-amino acrylic acid-pyridoxal phosphate complex. Production of ammonia and pyruvic acid could arise from the reaction of this complex with water. Such a mechanism would account for the failure of previous workers to find an intermediate in the enzyme reaction and also offer an explanation as to why indole production is not merely a reversal of the tryptophan desmolase system.

A Possible Model of the Tryptophan-Tryptophanase Action.

By J. W. Baker (introduced by F. C. Happold). (*Department of Organic Chemistry, The University, Leeds*)

There is some evidence that maximum activity in, for example, anti-bacterial (Albert, 1951) and oestrogenic (Clark, 1950; Rideal & Schulman, 1939) activity is closely associated with the 'area of flatness' of the molecule, which permits adsorption of the system on the substrate in a unimolecular layer. Such adsorption may involve functional groups (e.g. hydrogen bonding) and van der Waals forces, necessitating highly specific stereochemical requirements.

By the use of molecular models it is shown that (1) pyridoxal phosphate and tryptophan molecules fit neatly together to form a molecular complex in which the interacting aldehyde group of the former and the amino-group of the latter are brought into close juxtaposition, and (2) in this complex with L-tryptophan (but *not* with the D-form), the various groups through which, the results of Gooder & Happold (preceding abstract) suggest, bonding with the substrate occurs, all lie in a plane.

A tentative mechanism is indicated whereby decomposition of this one, common, molecular complex in three different ways could give rise, respectively, to indole fission, deamination and decarboxylation reactions.

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The Fate of Intravenously Injected ^{131}I-Labelled Antigens and 3:5-Diiodotyrosine in Immune and Normal Rabbits.

By A. E. Almond, G. E. Francis, J. D. Hawkins and A. Wormall. (*Department of Biochemistry and Chemistry, Medical College of St Bartholomew's Hospital, London, E.C. 1*)

In earlier investigations (Francis, Hawkins & Wormall, 1953) it was shown that intravenously injected horse serum γ-globulins, trace-labelled with ^{131}I, are more rapidly removed from the blood stream of rabbits if the animals have previously received immunizing injections of horse serum γ-globulins.

Similar studies have now been made with a more foreign antigen, highly iodinated proteins (labelled with ^{131}I), and here also a significant difference is found between normal rabbits and rabbits previously immunized against iodinated proteins. The intravenously injected antigen disappeared appreciably more rapidly during the first half-hour from the blood stream of the immune rabbits than it did in the case of normal rabbits, irrespective of whether the labelled antigen was iodinated bovine serum γ-globulin (the antigen used for immunization) or iodinated rabbit serum γ-globulin. In all these experiments the difference is most marked when only small amounts of antigen are injected into the blood stream.

Experiments have also been made with the corresponding hapten, 3:5-diiodotyrosine (labelled with ^{131}I). Removal of the intravenously injected hapten from the blood stream takes place more rapidly with rabbits previously immunized with iodinated bovine serum γ-globulin, the differences in the amount remaining in the blood being in these cases most noticeable in the period 1–5 days after the injections.

Evidence has been obtained which suggests that there may be fairly rapid liberation of relatively diffusible substances containing ^{131}I from highly iodinated proteins after their injection into the blood stream. It is advisable, therefore, to limit the use of these proteins, in investigations of this type, to short-term experiments.

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Phosphorus Metabolism in the Housefly *Musca domestica*.

By F. P. W. Winteringham, Patricia M. Loveday and G. C. Hellyer. (*Department of Scientific and Industrial Research, Pest Infestation Laboratory*)

For the purpose of studying the biochemistry of insecticidal action mechanisms *in vivo* the distribution of phosphorylated intermediates in normal and in poisoned flies is being studied. Adult flies are fed for 24 hr. with aqueous dextrose containing carrier-free $H_3{}^{32}PO_4$. They are then provided with aqueous dextrose only for a further period of 24 hr., subjected to mild cyclopropane anaesthesia, and immersed in liquid nitrogen. The chilled flies are washed free of external ^{32}P-contamination. After removing the appendages the muscle-rich thoraces are homogenized and extracted in an ethanol-formic acid-water mixture at -10°. Some of the washed insects have been sectioned and the histological distribution of the ^{32}P determined autoradiographically by a technique developed for water-soluble tracers (Winteringham, Harrison & Hammond, 1950). The phosphorus compounds present in the muscle

extracts (about 90 % of the total ^{32}P) are resolved by ascending unidimensional paper chromatography using an acetone-formic acid solvent (Burrows, Grylls & Harrison, 1952). Chromatography proceeds in 'evacuated' all-glass columns at 0° which ensures rapid and complete saturation with the solvent vapour. The strips are dried and radiometrically scanned automatically (Winteringham, Harrison & Bridges, 1952). In this way the labelled intermediates are determined quantitatively in samples equivalent to as little as $\frac{1}{100}$ part of the thoracic muscle of one fly. Two compounds, believed to be hexose phosphates, run together in this solvent. After location by scanning they are eluted quantitatively and concentrated on to a fresh strip of paper in one operation and resolved by running in an ammonia-methanol solvent (Burrows *et al.* 1952). On the basis of R_F values, rates of hydrolysis, etc. five of the separated components behave as ATP, two hexose phosphates, phosphoglyceric acid and orthophosphate. There is little difference in the ratios of the intermediates recovered whether the flies are killed (in liquid nitrogen) while in flight or under anaesthesia. The autoradiographs have demonstrated a high concentration of labelled material in the gut wall. Its composition appears to be the same as that found in the muscle. When the flies are killed by brief immersion in hot water the inorganic-phosphate/ATP ratio is greatly increased.

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The Stabilization of Elastin by a Polysaccharide. By G. C. Wood. (*Department of Medicine, University of Leeds*)

Elastic tissue (ox ligamentum nuchae) from which the collagen has been removed by treatment with boiling dilute acetic acid contains, interfibrillarly, a polysaccharide whose degradation products (sulphate, reducing material and amino sugar) appear when the tissue is dissolved under the action of elastase (Hall, Reed & Tunbridge, 1952). It was suggested that degradation of this polysaccharide might be essential for the dissolution of elastin. As a further indication of this, elastase has been separated into two fractions and indirect evidence suggests that one of these preferentially attacks the polysaccharide component of elastin (Hall, 1953).

Much of the polysaccharide may be extracted from collagen-free elastin by alkaline 10 % (w/v) solutions of calcium chloride and appears to be similar to chondroitin sulphuric acid (CSA) in that it contains sulphate, galactosamine and a uronic acid. It seemed of interest, therefore, to examine the action of elastase on CSA. The substrate was prepared from bovine nasal septa and the reducing material liberated when solutions were incubated with unfractionated elastase at 37° for various periods of time and over a wide pH range was estimated. There was no evidence from these experiments that the CSA was hydrolysed by the enzyme preparation.

In an attempt to obtain a measure of the cohesion of elastin, load/extension curves of strips of ox ligamentum nuchae were determined in the range 0–20 % extension in the direction of orientation of the fibrils. The curves are linear and unaffected by previous extension unless some treatment which decreases the cohesion of the material is interposed. The slope, $(df/dE)_a$, of the curves for strips from which extraneous protein and lipid have been removed and that, $(df/dE)_b$, of the curves for strips from which, in addition, collagen has been removed, were determined. Prolonged treatment with alkaline 10 % (w/v) solutions of calcium chloride at 37° have no effect on $(df/dE)_a$ or $(df/dE)_b$, although such treatment is known to remove much of the polysaccharide from finely divided elastin. Treatment with hyaluronidase at pH 5·2 has no effect on $(df/dE)_b$ but reduces $(df/dE)_a$ to a limited extent. Treatment with elastase preparations for various periods of time reduces both $(df/dE)_a$ and $(df/dE)_b$, ultimately to zero.

There is thus some doubt as to whether the polysaccharide present in elastin plays an important part in its stability. In any case it would appear to be something other than CSA.

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Studies on the Complex Nature of the Elastin-Elastase System. By D. A. HALL. (*Department of Medicine, Leeds University*)

Banga in 1951 expressed doubt as to the proteolytic activity of elastase. Hall, Reed & Tunbridge (1952) further suggested that the important enzymic process is mucolytic, while according to Lansing, Rosenthal, Alex & Dempsey (1953) elastase acts by liberating lipid from a lipoprotein complex. The present author has been unable to substantiate this, and has shown, moreover (1952), that the enzyme preparations used by Banga and by Lansing were possibly complex in nature, attacking more than one component of the substrate.

Fractionation of the enzyme with ammonium sulphate caused the precipitation of the activity over a considerable range of concentrations indicating the possibility of more than one component. There was, however, little evidence that fractions at different ammonium sulphate concentrations differed other than quantitatively in their activity. Slight separation of two components was observed after chromatography with ammonium sulphate solutions, but here the properties of the proteins appeared too alike to permit the use of the method for preparative purposes (see Hall & Wewalka, 1951). Similar results were observed on chromatography with aqueous acetone.

The most successful separation of the enzyme components has been obtained by adsorption on calcium phosphate and alumina. Batch experiments furnished two fractions. The substrates for testing these fractions were collagen free elastin preparations treated with alkali for varying periods of time. The two fractions appeared to be, respectively, rich in a component specific for the alkali labile fraction of the substrate, and in a component which preferentially attacked the residue after alkali treatment.

Although conclusive proof will have to await the isolation of two pure enzymes and two pure substrates there does appear to be definite evidence in favour of a complex system in which at least part of the substrate is mucoprotein in nature and the enzyme correspondingly mucolytic.

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Enzymic Estimation of Citric Acid. By S. DAGLEY and E. A. DAWES. (*Department of Biochemistry, University of Leeds, and Department of Biochemistry, University of Glasgow*)

The preparation of cell-free extracts that dissimilate citrate to pyruvate, acetate and CO_2 has suggested a method for citric acid estimation. Dagley & Dawes (1953) have shown that the enzyme is strongly developed when *Aerobacter aerogenes* is cultured anaerobically in media containing citrate limiting for growth. These conditions may be obtained by growth at 37° in 10 or 5 l. flasks filled to the neck with medium of the following composition: trisodium citrate dihydrate, 9 g.; $(NH_4)_2SO_4$, 1 g.; KH_2PO_4, 2 g.; and $MgSO_4$, $7H_2O$, 0·2 g. per l. When the cells had been harvested, about 10 g. cells paste were crushed in the Hughes (1951) press and taken up in phosphate buffer to provide 60 ml. of translucent extract after centrifugation. To 1 ml. of citrate solution was added 1 ml. of phosphate buffer (pH 7·0) containing 0·4 g./l. $MgSO_4 . 7H_2O$ and 1 ml. of extract, and after incubation at 37° for 15 min. 3 ml. of 10 % (w/v) trichloracetic acid solution was added and the pyruvate formed was determined by the method of Friedemann & Haugen (1943). For freshly prepared extracts a linear relation existed between citrate concentration and absorptiometer readings up to a concentration of 6×10^{-4}M of the former in the 3 ml. of incubation mixture; at higher concentrations the reaction ceases when conversion of citrate to pyruvate is not complete. Frozen extracts retained activity on storage but the extent

of the linear range of the calibration curve was reduced after freezing and thawing. Complete recovery was obtained for citrate added to peptone broth and the following compounds did not interfere with the determination: aspartic, glutamic, succinic, malic and fumaric acids, serine, glycine, alanine and phenylalanine. The method has been used to follow disappearance of citrate from bacterial culture media.

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Production of Keto Acids from Acetate by a Vibrio. By S. DAGLEY and M. D. PATEL. (*Department of Biochemistry, University of Leeds*)

Dagley, Dawes & Morrison (1951) harvested *Aerobacter aerogenes* from media supplying glucose and compounds of the tricarboxylic acid cycle as sole sources of carbon and aerated non-proliferating suspensions with acetate-phosphate solutions: pyruvate accumulation was only observed when the cells had previously utilized acetate for growth. We have extended these observations for a vibrio (Happold & Key, 1932) that utilizes a wide range of compounds in growth. Cells were harvested from defined media supplying the following as sole sources of carbon: citrate, succinate, malate, fumarate, mandelate, benzoate, *p*-hydroxybenzoate, phenylacetate and phenol. Suspensions of equal turbidities (0·05–0·1 mg./ml. dry wt.) were then made from each batch in growth media supplying (*a*) the original substrate, (*b*) acetate as sole carbon sources. Samples were withdrawn from (*a*) and (*b*) at suitable time intervals during growth and the cells, after separation by centrifugation, were aerated for one hour with acetate-phosphate solution. The total keto-acid concentration in the latter was then determined by the method of Friedemann & Haugen (1943). In this way it was possible to study the development of the ability of a given batch of cells to produce keto-acids from acetate. In medium (*b*) the ability was developed progressively during growth; but cells growing in (*a*) lacked this capacity in every case. The latter cells, however, were able to oxidize acetate readily in the Warburg respirometer; and further indication that keto acid excretion is to be associated primarily with growth processes rather than respiration was furnished by omitting the source of N from (*b*), when growth was precluded and development of the ability of the cells to produce keto acids from acetate was arrested.

Production of keto acids from acetate was observed for cells that utilized in growth fatty acids of chain length up to C_{10}. It may be suggested that β-oxidation occurred and that the vibrio developed a dicarboxylic acid cycle for C_2 fragments and acetate utilization. On the other hand cell-free extracts have been prepared, from cells grown on a number of carbon sources including fatty acids, that oxidize citrate with an uptake of two O per mole; whilst from chromatography the chief keto acid accumulating in acetate oxidation appears to be α-ketoglutaric.

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The Reactions of some Haemoglobin Derivatives with ^{131}I-labelled *p*-Iodophenylhydroxylamine and *p*-Iodonitrosobenzene. By H. JACKSON and R. THOMPSON. (*Christie Hospital and Holt Radium Institute, Manchester* 20)

p-Iodophenylhydroxylamine (IPhNHOH) and *p*-iodonitrosobenzene (IPhNO) have been shown to penetrate red cells with facility and combine firmly with the cell pigment (Crick & Jackson, 1953). Methaemoglobin is formed concurrently in the cells *in vivo* and *in vitro*, but only transiently in the former instance. The reformation of oxyhaemoglobin is not accompanied by loss of the combined iodo-compound

from the cell. With crystalline haemoglobin solutions, no initial formation of methaemoglobin has been observed.

The reactions of radioactive IPhNHOH and IPhNO with solutions of crystalline rat oxyhaemoglobin, carboxyhaemoglobin, methaemoglobin and alkaline haematin have now been examined and shown to take place in stoichiometric proportions. Phenylhydroxylamine (PhNHOH) and nitrosobenzene (PhNO) were previously known to react with oxyhaemoglobin (Heubner, Meier & Rhode, 1923; Jung, 1940). Keilin & Hartree (1943) studied the formation of the pigment complexes with haemoglobin and alkaline haematin, and concluded that reaction was due to nitrosobenzene and that phenylhydroxylamine underwent prior oxidation to this substance. Their spectroscopic examination of the action of oxygen, carbon monoxide and hydrosulphite on the pigment complexes led them to believe that the nitrosobenzene component was readily dissociated by the influence of these reagents, liberating unbound pigments. Whilst similar spectroscopic changes occur when IPhNHOH and IPhNO are used instead of PhNHOH and PhNO in the above reactions, the radioactive tracer reveals that molecular proportions of the iodo-compounds are, in fact, retained. Prior oxidation of the hydroxylamino-group to nitroso- is also not a necessary preliminary to reaction with haemoglobin pigments; for example, carboxyhaemoglobin reacts directly with IPhNHOH without change in absorption spectrum.

It is concluded that the reactions of IPhNHOH and IPhNO with various haemoglobin pigments are likely to be paralleled by PhNHOH and PhNO, and that spectroscopic data alone is inadequate in studies of these reactions.

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