PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **465th Meeting** *of the Society was held in the Pest Infestation Laboratory, London Road, Slough, Buckinghamshire, on Saturday,* 12 *November* 1966, *when the following papers were presented:*

COLLOQUIUM ON 'COMPARATIVE BIOCHEMISTRY OF RESISTANCE TO DRUG ACTION'

Enzyme Adaptation as a Mechanism of Drug Tolerance

By H. REMMER. (*Institut für Toxikologie der Universität Tübingen*)

Human beings and animals adapt themselves to drugs either by a decreasing sensitivity of the receptor site in the organism or by an accelerated breakdown of the active agent. Tolerance to morphine, pethidine, nicotine and other alkaloids is due to a diminished response of the cell, but not to an increased metabolism of the compound. However decreased sensitivity of the receptor in the CNS plays a minor role in the tolerance to most of the hypnotics. Their metabolism to ineffective metabolites can considerably increase if they are repeatedly used. Such drugs can be converted faster which are metabolized by hydroxylating enzymes in the liver cell. Those enzymes can be induced after the first dose of a lipid-soluble hypnotic. The hydroxylation rate increases because of cytochromal enzyme, which is completely unspecific and oxidizes numerous compounds foreign to the organism. Therefore the metabolism not only of the inducing drug but of all substances oxidized by these enzymes is increased after administration of hypnotics.

The inducing effect is not limited to hypnotics. Numerous lipid-soluble drugs, such as nikethamide, tolbutamide and antihistamines produce a similar induction of the drug-hydroxylating cytochrome.

After repeated doses of strong inducing agents such as phenobarbital, tolbutamide and nikethamide the amount of further enzymes located in the endoplasmic reticulum of the liver cell increases. Enzymes which have nothing to do with the breakdown of foreign compounds, however, are induced slightly or not at all. At the same time an augmentation of smooth membranes of the endoplasmic reticulum in the liver cell is seen with the electron-microscope. Morphologic changes in other cell structures are not observed. Chemical determinations of the constituents of smooth membranes isolated from the liver cells confirmed the two- to threefold increase of these membranes. The accelerated incorporation of labelled amino acids into proteins and of radiophosphate into lipids of liver microsomes is in accord with the proliferation of the smooth membranes, which lasts for 1–3 weeks after ending the treatment.

This form of adaptation which produces changes in the endoplasmic reticulum of the liver cell occurs in all mammalian species and explains the unspecific tolerance to drugs by an increased breakdown.

Biochemical Implications of 'Training' for Resistance in Bacteria

By A. C. R. DEAN. (*Physical Chemistry Laboratory, University of Oxford*)

'Training', i.e. serial subculture in medium containing increasing concentrations of drugs may be explained by two mechanisms which are not mutually exclusive. Either most of the cells undergo progressive modifications or a small proportion of resistant mutants is selected. Resistance of an unstable kind may occur after a period of growth in drug medium so short as to exclude any appreciable selection. A greater stability results from more prolonged training and in this procedure the growth rate improves up to a limit generally less than in the absence of drug.

The thesis that the cell contains a vast, closed, branched network of mutual dependencies has been elaborated elsewhere and a mathematical analysis predicts that in a given environment the ratios of the various components (nucleic acids, enzymes, etc.) will automatically adjust to those compatible with optimum rate of growth. The cell, however, is also organized in space and hence corresponding to the optimum network will be an optimum arrangement of the macromolecular constituents. Changes in the latter would be expected to occur more slowly than changes in the network and on this basis a satisfactory explanation of the stability of bacterial adaptations can be given (Dean & Hinshelwood, 1966). This need not necessarily conflict with mutation-selection theories since in examples where resistance clearly arises by this mechanism the mutants usually require a period of growth in drug

medium before the optimum rate is attained. The question is whether a preliminary change in the genetic code is a prerequisite or whether in some situations compensating adjustments in the enzyme balance of the cells can occur without it.

Various biochemical mechanisms leading to resistance can be envisaged. The susceptible enzyme(s) may expand to counteract the inhibition, or their affinity for the drug may become reduced. A less affected metabolic pathway may be brought into use or drug-destroying enzymes or other antagonists might be induced by the drug. These are all amenable to the thesis just outlined. On the other hand a mutation resulting, for example, in a loss of cellular receptors for the drug or a decreased permeability could also lead to resistance. Examples will be discussed.

Dean, A. C. R. & Hinshelwood, Sir Cyril (1966). *Growth, Function and Regulation in Bacterial Cells*, Oxford: Clarendon Press.

Biochemical Mechanisms of Bacterial Resistance to the Tetracyclines

By T. J. FRANKLIN. (*Imperial Chemical Industries Ltd., Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire*)

The first attempt to explain bacterial resistance to chlortetracycline in biochemical terms was made by Saz & Martinez (1956) who suggested that resistance in *Escherichia coli* was probably due to a loss in sensitivity to chlortetracycline of the enzyme nitroreductase. Inhibition of this enzyme in sensitive cells by the antibiotic was thought to be the principal cause of the bacteriostatic action of chlortetracycline.

More recent evidence, however, indicates that the tetracyclines probably owe their action to a direct inhibition of protein biosynthesis at the ribosomal level (Franklin, 1966).

Studies with a strain of *E. coli* bearing the transferable resistance factor to several antibiotics including the tetracyclines, suggest that in the case of the tetracyclines at least, resistance is probably attributable to an inhibition or loss of the active tetracycline concentrating mechanism which exists in sensitive cells (Izaki & Arima, 1963; Izaki, Kiuchi & Arima, 1966). Franklin & Godfrey (1965) found that the binding of labelled chlortetracycline and tetracycline by a resistant mutant of *E. coli* selected by serial passage through increasing concentrations of chlortetracycline was very much less than the binding by the sensitive parent strain.

Franklin, T. J. (1966). In *Biochemical Studies of Antimicrobial Drugs*. Sixteenth Symposium of the Society for General Microbiology p. 192. Cambridge: University Press.

Franklin, T. J. & Godfrey, A. (1965). *Biochem. J.*, **94**, 54.

Izaki, K. & Arima, K. (1963). *Nature, Lond.*, **200**, 384.

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Saz, A. K. & Martinez, L. M. (1956). *J. biol. Chem.* **223**, 285.

Biochemical Mechanisms of Insect Resistance to Anticholinesterases

By F. J. OPPENOORTH. (*Laboratory for Research on Insecticides, Wageningen, The Netherlands*)

Resistance to anticholinesterases in insects is essentially the result of selection of biochemically aberrant individuals, which pre-existed in the original population. Oligogenes have been found in many cases to be responsible for an important part of the resistance.

There are two important classes of anticholinesterases in use as insecticides: organophosphorus (OP) compounds and carbamates. Biochemical studies so far have been mainly concerned with the former.

Resistance to the OP compounds can be effected by different mechanisms. In the spider mite, *Tetranychus urticae*, my colleague Smissaert (1964) found a greatly reduced rate of inhibition of cholinesterase by OP compounds. Bimolecular rate constants are 10^2 versus 10^5 with paraoxon, 2×10^4 versus 3×10^6 ($l\,mole^{-1}\,min.^{-1}$) with diazoxon for R and S strains respectively. This difference between the cholinesterases is due to a single gene mutant, causing a high degree of resistance to many cholinesterase inhibitors. Interestingly, the mutant displays a threefold reduction of activity to acetylcholine, but apparently this is quite compatible with life. A similar resistance mechanism has been found in the tick, *Boophilus microplus* (Lee & Batham, 1966).

The housefly, *Musca domestica*, relies mainly on detoxication mechanisms in its resistance to anticholinesterases. One type of OP-resistance is associated with a virtual loss of activity of a certain aliesterase. In resistant strains this enzyme is replaced by phosphatases which hydrolyse the OP-inhibitors. At least three different phosphatases derived from the aliesterase occur in different resistant strains under the control of alleles of the same gene. Hydrolysis by these phosphatases is characterized by extremely low K_m values and turnover rates (e.g. 10^{-8} M and 0·25 min^{-1} for paraoxon) (van Asperen, 1964; Oppenoorth, 1965).

Recently we found that other kinds of OP resistance are counteracted by methylenedioxy-compounds, and are therefore thought to be due to oxidative degradation of the OP compound. The presence of different cross-resistance patterns and differences in genetic background show that increased detoxication of this type can be caused by

different mechanisms. These mechanisms are also responsible for cross-resistance to unrelated insecticides (DDT, carbamates).

Several other OP-degrading enzymes have been found in R strains of insects. The examples mentioned should suffice to show the great variety of changes by which insects can adapt to the altered chemical environment.

Lee, R. M. & Batham, P. (1966). *Entomol. Experimentalis et Applicata*, **9**, 13.
Oppenoorth, F. J. (1965). *Ann. Rev. Entomol.* **10**, 185.
Smissaert, H. R. (1964). *Science*, **143**, 129.
van Asperen, K. (1964). *Entomol. Experimentalis et Applicata*, **7**, 205.

Biochemical Implications of Resistance in Diptera to the Cyclodiene Insecticides

By G. T. Brooks. (*Biochemistry Department, Pest Infestation Laboratory, Slough*)

Though the cyclodiene insecticides appear to act as central nerve poisons, there is no precise knowledge of their mode of action. Moreover, the commercial cyclodienes such as dieldrin appear to be remarkably stable in both normal and resistant Diptera, while most mechanisms of resistance to toxicants appear to involve, at least partly, some recognizable metabolic detoxication.

However, in contrast to the complexity of resistance to some other insecticides, the inheritance of resistance to cyclodienes appears to be relatively simple. The cyclodiene cross resistance spectra of a number of species are similar and in an increasing number of cases a single gene of intermediate dominance appears responsible for the resistance (Busvine, 1964; Guneidy & Busvine, 1964). This uniformity suggests an equally simple physiological alteration in resistant strains. A single resistance mechanism might involve reduced sensitivity of the central nervous system to the toxicant, or a low rate of detoxication at some vital site.

Available evidence suggests the first mechanism but Gerolt (1965) demonstrated a low level of metabolism of sublethal doses of dieldrin in both susceptible and resistant houseflies, so that metabolic pathways do exist for dieldrin. Their significance for resistance is questionable. The stability in Diptera of dieldrin and other commercial cyclodienes is exceptional; the natural resistance of houseflies to many related compounds is largely associated with detoxications effected by microsomal enzymes (Brooks, 1966). Certain inhibitors of microsomal oxidation considerably increase the toxicity of many of these compounds to normal but not to dieldrin resistant houseflies. These inhibitors do not increase dieldrin toxicity to resistant houseflies selected with dieldrin, indicating that oxidative detoxication is not a resistance factor, though it can be argued that the inhibitor may not be reaching the detoxicating enzymes.

Schonbrod, Philleo & Terriere (1965) showed, however, that a housefly strain selected for naphthalene resistance was twice as tolerant to this compound as a susceptible strain, had an increased ability to hydroxylate naphthalene and was virtually immune to dieldrin. Consequently, they implicated hydroxylation in dieldrin resistance. Whether inhibitors of naphthalene hydroxylation also suppress this associated dieldrin resistance is unknown. A careful examination of different species may yet reveal examples in which dieldrin detoxication is highly developed.

The biochemical implication of the uniform cross resistance spectra of Diptera to cyclodienes is unknown, but the observation (Brooks & Harrison, unpublished) that δ-chlordane* (Büchel, Ginsberg & Fischer, 1966), stereochemically analogous to isobenzan (1,3,4,5,6,7,8,8-octachloro-1,3,3a,4,7,7a-hexahydro-4,7-methanoisobenzofuran), is likewise toxic to dieldrin resistant houseflies, while cross resistance to the position isomer β-chlordane (Büchel *et al.*, 1966) is complete, raises interesting questions regarding stereochemistry and metabolism.

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Guneidy, A. M. & Busvine, J. R. (1964). *Bull. ent. Res.* **55**, 499.
Schonbrod, R. D., Philleo, W. W. & Terriere, L. C. (1965). *J. Econ. Entomol.* **58**, 74.

* Gift of Shell Research, Birlinghoven.

COMMUNICATIONS

The Incorporation of 2-Aminobutanols into the Phospholipids of Housefly (*Musca domestica*) Larvae

By R. G. Bridges and J. Ricketts.* (*Biochemistry Department, A.R.C. Pest Infestation Laboratory, London Road, Slough, Bucks.*)

2-Aminoalcohols (aminopropan-1-ol to aminohexan-1-ol) when added to a casein diet (Bridges, Ricketts & Cox, 1965) at 16·0 μmole/g. casein partially replace the choline and ethanolamine of the phospholipids of larvae grown on this diet. When no choline is included in the diet some of the aminoalcohols cause retardation of the growth of the larvae. 2-Aminobutan-1-ol and 2-amino-2-methylpropan-1-ol have the greatest effect and cause death of the larvae before the sixth day after hatching. If choline chloride is added to the diet at the same concentration along with the aminoalcohols the growth of the larvae is normal.

Reduction of the choline level in the diet to 0·25 μmole/g. has no marked effect on growth but lowers the level of phosphatidylcholine in the larvae from 17–4% of the total phospholipid. With aminobutanols in the diet a marked inhibition of growth occurs in diets containing between 1·0–0·5 μmole choline/g. (2-aminobutan-1-ol) and between 0·5–0·25 μmole choline/g. (2-amino-2-methylpropan-1-ol). Decreasing the level of choline in the diet causes an increase in the proportion of phosphatidylaminobutanols found in larval lipids. This rise is most marked with 2-amino-2-methylpropan-1-ol, the phosphatidyl-derivative increasing from 7–24% of the total phospholipid.

A series of 2-*N*-monoalkylaminobutan-1-ols has been prepared. These compounds when fed to larvae in the absence of choline do not retard larval growth to the same extent as does 2-aminobutan-1-ol. Most are found to become incorporated into phospholipid and some dealkylation occurs with the formation of phosphatidyl-2-aminobutan-1-ol. The 2-*N*-dimethyl-derivatives and the fully methylated quaternary salts of the two aminobutanols, although incorporated into phospholipids in the absence of choline in the diet, do not cause any inhibition of growth.

After examination of a trichloroacetic acid extract of larvae grown on a medium containing 2-aminobutan-1-ol and choline (both at 16·0 μmole/g.) the following distribution has been determined expressed as μmole/g. wet weight: 2-aminobutan-1-ol 3·3, 2-aminobutan-1-ol phosphate 5·7, lipid-bound 2-aminobutan-1-ol 2·8, and a small fraction (0·1) tentatively identified as the cytidinediphosphoryl-derivative.

* Present address: Courtauld Institute, Middlesex Hospital, London, W. 1.

Bridges, R. G., Ricketts, J. & Cox, J. T. (1965). *J. Insect Physiol.* **11**, 225.

The Calcium-stimulated Incorporation of Isotopic Serine and Ethanolamine into the Phospholipids of Housefly (*Musca domestica*) Larvae

By H. D. Crone. (*Biochemistry Department, A.R.C. Pest Infestation Laboratory, London Road, Slough, Bucks.*)

The incorporation of [2-^{14}C]ethanolamine and L-[3-^{14}C]serine into the phospholipids in homogenates of the fat bodies of larval houseflies has been studied. This incorporation is dependent on the calcium ion concentration, maximal rates being obtained at 20 mM in imidazole buffer. Magnesium will not replace calcium, but is not directly inhibitory. Manganese, zinc and mercuric ions at 10 mM completely inhibit incorporation. The pH optimum in veronal buffer lies between 7·25 and 8·25, beyond these points the activity falls off sharply. The incorporation is greatly reduced by 1 mM-cetyltrimethylammonium bromide and by 0·1% Triton X-100. Serine and ethanolamine both have K_m values of 2×10^{-4} M when measured in imidazole buffer with 10 mM calcium.

The incorporation of both compounds is competitively inhibited by tris buffer (Crone, 1966) and by a number of other aminoalcohols. Effectiveness as competitors depends on the distance apart of amino and hydroxyl groups (2-aminopropan-1-ol is better than 4-aminobutan-1-ol) and on the possession of a primary hydroxyl group (2-aminopropan-1-ol is better than 1-aminopropan-2-ol). *N*-Monomethyl and *N*-dimethylethanolamine are indistinguishable from ethanolamine or serine in their effect, but choline is less effective as a competitor.

The incorporation of the radioactive serine or ethanolamine is judged to be a direct exchange with existing nitrogenous bases on the phospholipids, rather than a reflexion of net synthesis of the lipids. This is because of the kinetics of the incorporation, which do not favour the presence of intermediates, and because the incorporation is calcium dependent, whereas the incorporation of [^{32}P]phosphorylethanolamine in the same system is stimulated by magnesium and not by calcium.

Similar calcium-stimulated exchanges have been reported to occur in mammalian tissue by Artom (1961), Borkenhagen, Kennedy & Fielding (1961) and by Hübscher (1962). Hübscher obtained evidence for several distinct systems exchanging various compounds (serine, choline and inositol) with unknown phosphatide acceptors. In the present work the results are in accord with the presence of one enzymic system exchanging ethanolamine, serine and foreign aminoalcohols with the bases of phospholipids, the precise nature of which has not been elucidated. Bridges & Ricketts (1966) have reported the incorporation of several unnatural aminoalcohols into the phospholipids of housefly larvae *in vivo*, and it is hoped to discover whether the calcium-stimulated exchange is involved in this.

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Crone, H. D. (1966). *Biochem. J.* **100**, 12P.
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Aspects of Dithionate Metabolism in *Thiobacillus denitrificans*

By W. K. T. Cook (introduced by T. J. Bowen). (*Department of Biochemistry, University of Leeds*)

It has been reported (Lieske, 1912) that dithionate was metabolized by *Thiobacillus denitrificans*. Owing to the difficulty of detecting dithionate, work on this compound has been somewhat neglected. A sensitive test for dithionate on paper chromatograms has recently been described (Bowen & Cook, 1966) which has enabled us to make further studies in this field.

The mixture of nucleotides produced in the preparation of adenosine 5′-phosphosulphate (APS) (Cherniak & Davidson, 1964) was examined chromatographically and the presence of a new nucleotide was detected. After isolation and analysis this was found to have a phosphorus to sulphur ratio of 1:2, and this was confirmed by using ^{35}S labelled material. The infrared spectrum showed an absorption band typical of dithionate while the ultraviolet spectrum resembled that of adenosine. We have tentatively named this compound adenosine 5′-phosphodithionate (APSS).

APSS was detected chromatographically when crude extracts of *T. denitrificans* were incubated in the presence of AMP, sulphite and ferricyanide. Radioautography showed the production of APSS from APS and sulphite in the presence, and absence, of ferricyanide. Whole-cell cultures of *T. denitrificans* were observed to produce and metabolize dithionate in the presence of thiosulphate and preliminary experiments indicated that dithionate was produced from APSS.

When the bacteria were grown on a medium containing thiosulphate and dithionate there was a marked increase in bacterial protein coupled with a decrease in turbidity due to decreased precipitation of sulphur: there was no change in the utilization of thiosulphate.

On the evidence available it is suggested that dithionate is a metabolite of *T. denitrificans* and that APSS is implicated in its metabolism. There are indications that there are other sulphur-containing nucleotides which may also be implicated.

We are grateful to Professor F. C. Happold for advice and to Professor J. Baddiley for helpful correspondence. One of us (W.K.T.) acknowledges support from the S.R.C.

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A Major Protein Constituent of Pupae of the Blowfly *Calliphora erythrocephala* (Diptera)

By E. A. Munn, A. Feinstein and G. D. Greville. (*A.R.C. Institute of Animal Physiology, Babraham, Cambridge*)

When pupae of *C. erythrocephala* are squashed in dilute salt solutions and the extract examined in the electron microscope by the negative staining technique, little is seen save large numbers of fairly symmetrical particles about 100 Å across. Similar particles are seen when feeding larvae, prepupae and newly emerged adults are examined.

The particles have been isolated in pure form. The soft parts of 6-day pupae were crushed in a solution (pH 6·3) containing 0·13 M-NaCl, 17 mM-phosphate, 10 mM-$MgCl_2$ and 4 mM-HCN. The fluid, after straining, was centrifuged to remove nuclei, mitochondria and ribosomes. The particles were completely sedimented in 4 hr. at 160000 *g*. They were resuspended and centrifuged again. Preparations at this stage showed a symmetrical peak in the analytical ultracentrifuge but contained a more slowly sedimenting contaminant to the extent of 7% or less. The material was further purified by density-gradient centrifugation and zone electrophoresis on Pevikon powder (Shandon Scientific Co.). It now showed a single component in the ultracentrifuge with $S^0_{20,w} = 20$S. It gave a single spot when stained for protein after agar-gel electrophoresis. Immunoelectrophoretic patterns,

developed with a rabbit antiserum to a crude preparation, consisted of several arcs before density-gradient centrifugation; the final product gave only one, and this was associated with the stained spot.

At 0·75% concentration, $S_{20,w}$ was 18·3s and $D_{20,w}$ $3·2 \times 10^{-7}$ cm.^{2} sec.$^{-1}$, indicating a molecular weight of $5·1 \times 10^5$ and a compact symmetrical structure, in agreement with the electron-microscopic appearance. As the pH is raised above 6·3, the particles increasingly dissociate reversibly into more slowly sedimenting units. An amino-acid analysis will be reported; the protein contains no cysteine or cystine and has an unusually high tyrosine content, reflected in a high $E_{280\,m\mu}/E_{250\,m\mu}$ ratio (3.3).

We suggest that the protein be called *calliphorin*, until such time as this may be proved unsatisfactory.

Two Distinct Low-Molecular-Weight Ribonucleic Acids Localized Within the Chloroplasts of *Vicia faba* Leaves

By T. A. Dyer and Rachel M. Leech.* (*A.R.C. Unit of Plant Physiology and Department of Botany, Imperial College, London, S.W.* 7)

The view that chloroplasts are genetically autonomous is supported by any demonstration that their nucleic acids are unlike those of the rest of the cell. In leaves dissimilarities between chloroplast and nuclear DNA (see Green & Gordon, 1966 for summary) and between chloroplast and cytoplasmic ribosomes (Lyttleton, 1962; Sissakian *et al.* 1965) have been reported. To determine whether the low molecular weight RNA components of the chloroplast are also distinct from those of the rest of the leaf cell, the RNA of whole leaves and subcellular organelles were examined.

Structurally intact chloroplasts were isolated according to Leech (1964). Ribosomes were sedimented by centrifugation at 105000*g* for 6hr. Chloroplast ribosomes were derived from osmotically ruptured intact chloroplasts and 'cytoplasmic' ribosomes from the $40000 \times g$ supernatant of a leaf brei prepared to avoid rupture of the larger cell organelles.

Nucleic acids, extracted in the cold in 6% 4-aminosalicylate and phenol–water (80:20w/w) in a ratio by volume of 1:2, were precipitated from the aqueous phase by the addition of 2 volumes of ethanol and standing for 4 hr. at −20°. The nucleic acids were chromatographed on methylated serum albumin–kieselguhr (MAK) columns as described by Sueoka & Yamane (1962), using NaCl gradients 0·3–1·15M. The adsorption of the column effluents was continuously recorded at 254mμ.

The elution profiles revealed a low molecular weight RNA in the 'cytoplasmic' ribosomes from *Vicia faba* leaves and a similar, but not identical, component was present in chloroplast ribosomes. The identity of the corresponding components in the total leaf homogenate was established by co-chromatography.

The elution profiles of 'cytoplasmic' soluble RNA and chloroplast soluble RNA show the presence of only one peak in the former but two in the latter, i.e. the chloroplasts contain a unique soluble RNA in addition to the distinct low molecular weight RNA associated with the chloroplast ribosomes. Preliminary studies of amino-acyl-RNA formation by chloroplasts indicated that the transfer activity is associated with the first eluting chloroplast component. The dispersed elution of both components suggests that they are heterogeneous. Each nucleic acid component was identified in at least five separate preparations, the profiles were completely distinct from those resulting from ribonuclease activity and the additional peaks of the chloroplasts could not have been due to bacterial contaminations. The bacterial count of the chloroplast preparations was $5 \times 10^3 - 10^3$/ml. (0·15mg. chlorophyll).

In roots of *Vicia faba* only the cytoplasmic soluble RNA component and the low molecular weight RNA associated with cytoplasmic ribosomes is present: the two distinct low molecular weight RNAs characteristic of the chloroplast are absent.

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* Present address: Department of Biology, University of York, Heslington, York, England.

The Rapid Evaluation of Radio Chromatograms Using a Spark Chamber

By B. R. Pullan, R. Howard, I. Kaye and H. Lowe (introduced by K. R. L. Mansford) (*Department of Physics, St George's Hospital, London, S.W.*1 and *Department of Biochemistry, Imperial College, London, S.W.* 7)

A spark chamber can produce a visible electrical discharge on or near to the path of a β particle and if operated in a self-triggered manner can be used as a radiation detector. Such a detector placed over a radio chromatogram will spark at the positions at which β-particles pass through it and if these sparks are photographed so that their images are allowed to add together, a picture similar to an autoradiograph can be obtained but in a much shorter time.

The spacial resolution of present chambers is of the order of 0·5cm. and a spot of carbon 14 of 0·5cm. diameter and of 0·03mμc activity can be visualized with a ten-minute exposure; a similar spot of Tritium of activity of 0·3mμc can also be visualized with the same exposure. The spark chambers have been designed (Pullan, Howard & Perry, 1966) so that it is possible to extract numerical data on the distribution of sparks, and equipment has been constructed so that spark co-ordinates can be recorded on paper tape for subsequent computer analysis.

Spark chambers are particularly suitable for the evaluation of radio chromatograms on paper or thin layer, in one or two dimensions because they can record data in both visual and numerical form from the whole of a chromatogram at once with good spacial resolution in two dimensions and with a high efficiency for detecting β particles.

Pullan, B. R., Howard, R. & Perry, B. J. (1966). *Nucleonics*, **24**, no. 7, 72.

Urinary Indoles of Primates—A Comparative Study

By I. Smith.* (*University of Texas Dental Branch, Houston, Texas, U.S.A.*)

It is being currently held that biochemical experimentation on primates must throw light on human metabolism. Thus a great deal of work has been done on the attempted production of phenylketonuria (Harlow & Waisman, 1965) and other diseases in monkeys, and primates have been used for experiments which would be considered unethical in man. Underlying all this work is the tacit assumption that all primates possess similar or identical metabolism and the aim of this work was to determine the validity of this assumption.

Urine and blood was collected from a variety of primates and examined without pre-treatment for indoles. Whole urine (100–250μl.) was chromatographed by a two-way paper-chromatographic procedure (Jepson, 1960) which was found superior to thin layer methods. Whole blood (10μl.), or serum, was examined using a two way paper electrophoresis-chromatography procedure (Smith, 1966.)

Normal urinary indoles (i.e. Ehrlich-positive reactors) patterns were obtained for 16 genera, all of whom showed genus-specific patterns. Man exhibits a normal pattern of urea plus indoxyl sulphate, which is a gut-bacterial metabolite, together with occasional traces of tryptophan. However, other species show up to ten separate spots ranging through the usual purple, blue, pink, red and yellow colours. The only compound common to all urines was urea and quantities of urine were chosen to give similar amounts of this on all chromatograms. Indoxyl sulphate was absent from some species and a blue spot (6-sulphatoxyskatole?) was present in a number of species taxonomically close to the rhesus monkey but absent from others more distant. Blood showed no Ehrlich reactors. Whatever the chemical nature of the compounds located, they must be metabolites of the dietary indolic-amino acid tryptophan. A number of points follow from this. First, there is a possible basis for primate biochemical taxonomy using urinary small molecules instead of the serum proteins. Second, it is reasonable to suppose that the metabolism of other aromatic amino acids may vary amongst the primates. Hence data obtained from such primate experimentation should be interpreted with caution when applied to experimental medicine and human diseases involving derangements in aromatic metabolism.

* Present address: Courtauld Institute, Middlesex Hospital, London, W. 1.

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Smith, I. (1966). In press.

Parallelism between the Effects Exercised by Hepatotoxic Agents on Microsomal Phosphatases in Rat Liver

By G. Feuer, L. Golberg, and A. Hunt. (*British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey*)

Liver glucose 6-phosphatase and inorganic pyrophosphatase are very sensitive to the influence of hepatotoxic agents (Feuer, Golberg & Le Pelley, 1965; Feuer, Golberg & Gibson, 1965). A further group of microsomal enzymes, the nucleoside diphosphatases, have now been studied. In rats given coumarin (50–200mg./kg. orally daily for 7 days) or carbon tetrachloride (0·5ml./kg. as a single subcutaneous dose) reduction of glucose 6-phosphatase and inorganic pyrophosphatase was accompanied by parallel increases in the activities of uridine, inosine and guanosine diphosphatases, but cytidine diphosphatase and thiamine pyrophosphatase were less affected. Similarly the increased

activities of glucose 6-phosphatase and pyrophosphatase induced by fasting or triamcinolone (10mg./kg. intraperitoneally daily for 5–7 days) were paralleled by decreases in nucleoside diphosphatases. In rats whose liver microsomal processing enzymes had been stimulated by butylated hydroxytoluene (Gilbert & Golberg, 1965) or phenobarbitone there was a greatly reduced effect of coumarin or carbon tetrachloride on the nucleoside and other microsomal phosphatases referred to above.

Phospholipases A, C or D acting on liver microsomes *in vitro* reduced glucose 6-phosphatase and pyrophosphatase and increased nucleoside diphosphatases, as hepatotoxins had done *in vivo*. Contrary to the observations of Ganoza (1964), added phospholipid from liver microsomes (untreated or coumarin-treated rats), or brain or egg phospholipids did not return enzyme activity to the original level before phospholipase action.

Treatment of microsomal preparations *in vitro* with Triton X-100 elevated the activities of glucose 6-phosphatase and pyrophosphatase to the levels attained with fasting or triamcinolone *in vivo*, which were not further raised *in vitro* by Triton; likewise, the reduced activities in microsomes from rats treated with coumarin were restored to the same high levels. These findings are in accordance with the observations reported by Nordlie, Arion & Glende (1965). In the case of nucleoside diphosphatases, the enhanced activities resulting from hepatotoxic action as well as the activities in microsomes from untreated rats, were both raised to the same level by Triton.

It is concluded that the changes in microsomal enzyme activity probably involve largely reversible alterations in the state of binding of each enzyme to microsomal phospholipid, or conformational changes in the enzyme occasioned by effects on the associated phospholipid, rather than activation or inactivation of the enzymes by destructive changes in the endoplasmic reticulum.

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A K9 Specific Polysaccharide of *Escherichia coli*

By A. P. MacLennan, E. C. A. Seneviratne and D. C. Hawkins. (*Microbiological Research Establishment, Porton, Wilts.*)

Many strains of *Escherichia coli* possess K (capsular) antigens (Kauffmann, 1954). These substances prevent the agglutination of cells by antibodies directed against the underlying O-antigen. More than 80 K antigens have been defined serologically and several have now been isolated and characterized (see Jann, Jann, Ørskov, Ørskov & Westphal, 1965).

A 'K9-specific serum' was prepared by absorbing a rabbit antiserum against *E. coli* serotype 09K9H12 with appropriate 09 and H12 strains.

K9 polysaccharide was prepared from 09K9H12 cells by Cetavlon/NaCl fractionation of phenol extracts, following precisely the procedures described by Jann *et al.* (1965). The serologically active Cetavlon Fraction 2 (Jann *et al.*, 1965) was chromatographed on a column of A50 DEAE-Sephadex in tris/NaCl buffer at 4° (0·05M-tris/0·025M-HCl/0·05M-NaCl, pH 8·3 at 22°). Elution by stepwise increase of NaCl concentration yielded a major fraction emerging in 0·2M-NaCl. This was rechromatographed with smaller increments of NaCl concentration. A fraction (Pool 4C) eluted in 0·175M-NaCl was further examined.

Pool 4C precipitated with 'K9-specific serum' and reduced its agglutination titre for 09K9H12 cells. In passive haemagglutination tests Pool 4C inhibited the agglutination by this serum of erythrocytes sensitized with Cetavlon Fraction 2. Comparative haemagglutination tests with 09 antiserum and red cells sensitized with 09 lipopolysaccharide showed the absence of 09 specificity from the K9 preparation. Heating (100°/2hr.) destroyed the serological activity of Pool 4C.

Analysis of Pool 4C gave galactose (38%), hexosamine (19·5%) and sialic acid (27%, as *N*-acetylneuraminic acid, by direct Ehrlich). Some, at least, of the hexosamine is *N*-acetylated. The values correspond with a molar ratio of 2:1:1.

The presumptive sialic acid component was not released by *Vibrio cholerae* neuraminidase. Moreover, a low value for sialic acid content (5·4%) was obtained by the periodate-thiobarbituric acid test (Warren, 1959), employing the recommended hydrolysis procedure for mucoproteins (0·1N-H_2SO_4/80°/1hr.). A higher value (12·5%) was obtained when H_2SO_4 concentration was increased to 2N, despite the considerable destruction of free sialic acid under these conditions.

E. coli 09K9H12 cells contain a second component with K9 specificity. This material is concentrated

in Cetavlon Fraction 3, and is further distinguished from Pool 4C by its thermostability.

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The Metabolism of a Lipid Peroxide

By I. P. Freeman and P. J. O'Brien. (*Department of Medical Biochemistry, Birmingham University*)

It was previously reported that most of the radioactivity of the [1-^{14}C]linoleic acid hydroperoxide (LAHPO) fed (1mg.) or injected intramuscularly (0·5mg.) to rats of weight 200–280g. was readily incorporated into tissue lipids. The distribution amongst the various lipid classes was similar to that expected for an unoxidized fatty acid (Freeman, 1964).

A liver triglyceride fraction was hydrolysed and the ethereal extract chromatographed by thin layer. Autoradiographs indicated that the radioactivity was distributed as 42% free fatty acid, 3% hydroperoxide, 25% as oxidized breakdown products and 31% base-line material (polymeric breakdown products, monoglycerides). The distribution was independent of whether the LAHPO was fed or injected. Radio-gas chromatography showed no C_{18} acid in the free fatty acid fraction. It can therefore be concluded that the metabolism of LAHPO does not involve direct conversion into linoleic acid but rather to intermediates which are metabolized by a mechanism similar to that for an unoxidized fatty acid.

Thin-layer radioautographs of extracted decomposition products formed by incubating aqueous [1-^{14}C]LAHPO with intestinal mucosa, liver mitochondrial or microsomal fractions were similar to those formed after incubation with cytochrome *c* or ferrous sulphate. The amount of decomposition was dependent on the concentration of the fraction, was hardly affected by EDTA or by previous boiling of the fraction, but was inhibited 50% by *p*-chloromercuribenzoate. Decomposition was increased by adding GSH or ascorbic acid. These results suggest that the intracellular decomposition of LAHPO is non-enzymic and involves haemoproteins and thiols.

To determine the nature of the decomposition products and thus possibly the decomposition mechanism involved, ether-soluble decomposition products were extracted after incubating large amounts of LAHPO with ferrous sulphate. These were separated on a 1mm. thick silica layer and extracted with ethanol. Each product was examined by u.v.-spectrophotometry, thin-layer chromatography and gas chromatography before and after different chemical modifications and compared with model compounds. Amongst the acids tentatively identified were dicarboxylic, epoxy, keto, mono and dihydroxy acids, as well as mixed esters of these acids, semialdehydes and polymeric material of high polarity.

Thin-layer autoradiographs of decomposition products of LAHPO after incubation with liver homogenate or supernatant fraction suggest a different mechanism of decomposition since the hydroxy acids and not the polymeric products are the final products formed.

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Lipid Peroxide in a Peroxidase-type Reaction

By P. J. O'Brien. (*Department of Medical Biochemistry, Birmingham University*)

Pure linoleic acid hydroperoxide (LAHPO) was prepared (O'Brien & Frazer, 1966), and the rate of degradation of aqueous solutions of LAHPO at pH 8·5 by haem catalysts was studied by measuring the extinction decrease at 232mμ. The rate was found to be first order with respect to LAHPO and catalyst concentrations, and for cytochrome *c* and haemoglobin was maximal at pH 3·0. At pH 3 the rate was 3·0 times that at pH 8·5.

At pH 8·5 the catalytic activities of haematin and haemoproteins decreased in the order methaemoglobin, haematin, cytochrome *c*, oxyhaemoglobin and peroxidase. It was previously reported that at pH 8·5 long-chain fatty acids, anionic and cationic detergents increased up to 6-fold the amount of LAHPO degraded by cytochrome *c* in the first minute (O'Brien, 1964). Protease treatment was found to cause a greater stimulation. The increased catalytic activity may be due to partial unfolding of the protein moiety around the haem. A loosening of haem-protein coordination bonds probably also occurs at pH 3 (Flatmark, 1965).

At pH 8·5, cytochrome *c* was 3 times less damaged by LAHPO than at pH 2·6 (O'Brien, 1966*a*) or at pH 8·5 in the presence of fatty acids (O'Brien, 1966*b*). Presumably, when the protein is partially unfolded, the haematin group is more easily damaged by the oxidizing species formed on decomposing LAHPO.

The rate of ascorbic acid oxidation by LAHPO and cytochrome *c* was also optimal at pH 3, but more pH-dependent than LAHPO decomposition. Because of the u.v.-absorption of LAHPO, and

difficulties in measuring the rapid initial rate, the kinetics were investigated with H_2O_2 instead of LAHPO. The oxidation of ascorbic acid after a small initial lag period was first order with respect to peroxide and cytochrome concentrations. In a double reciprocal plot the rate was proportional to the ascorbic acid concentration. No cytochrome destruction by H_2O_2 occurred until all the ascorbic acid had been oxidized.

The catalytic activity of haematin or trypsin-treated cytochrome *c* for ascorbic acid oxidation by LAHPO, however, increased as the pH was increased. After modification by proteases or protein denaturants, the greatly enhanced activity of cytochrome *c* at neutral pH is probably determined by its activity for decomposing LAHPO, and by the pH optima shift.

Uric acid and NADPH showed similar kinetics and behaved similarly to ascorbic acid in this peroxidase-type reaction.

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Oxidation of Small Thiols by Lipid Peroxides

By C. Little and P. J. O'Brien. (*Department of Medical Biochemistry, Birmingham University*)

Previous work (Little & O'Brien, 1966*a* & 1966*b*) showed that the oxidation of GSH by hydrogen peroxide or lipid peroxide was stimulated by the presence of cytochrome *c* and was approximately first order with respect to the peroxide concentration. The kinetics of thiol oxidation by lipid peroxides have been examined further.

Using linoleic acid hydroperoxide (LAHPO), the rate of catalysed oxidation of small thiols by LAHPO was found to obey the rate equation:

$$\frac{-\mathrm{d(SH)}}{\mathrm{d}t} = K(\mathrm{LAHPO})\,(\mathrm{catalyst}).$$

Adding EDTA, but no catalyst, severely inhibited the reaction, and the kinetic equation became:

$$\frac{-\mathrm{d(SH)}}{\mathrm{d}t} = K'(\mathrm{LAHPO})\,(\mathrm{SH}).$$

For monothiol oxidation, both the catalysed and the uncatalysed rates were stimulated as the pH increased, and also, at constant pH, thiols with the lowest values of pK_{SH} were oxidized most rapidly. It was therefore concluded that the mercaptide group was oxidized much more rapidly than the unionized thiol group.

The rate of uncatalysed oxidation of vicinal dithiols was much greater than for monothiols, probably because of the ease of disulphide formation in dithiols. The rate of haemoprotein-catalysed *vic*-dithiol oxidation had an acid pH optimum, whilst the haematin-catalysed reaction had an alkaline pH optimum. Thus, in this respect dithiols resemble most hydrogen donors (O'Brien, 1966). However, the kinetics of catalysed or uncatalysed dithiol oxidation by LAHPO resembled those shown by monothiols.

For the oxidation of GSH at pH 8·5, the activities of catalysts decreased in the order haematin > oxyhaemoglobin > myoglobin > cytochrome *c* > methaemoglobin. However, the catalytic activity of the cytochrome was stimulated by the protein denaturants urea (up to 1·5-fold) and sodium dodecyl sulphate (up to 1·7-fold), and also by previous digestion with proteases (up to 7·6-fold). The activity of a catalyst was not related to its redox potential or to its activity for LAHPO decomposition (O'Brien, 1966). It is likely therefore that the rate of SH oxidation was not related to the rate of LAHPO decomposition.

Prolonged incubation of GSH at pH 8·5 with an amount of LAHPO sufficient to cause 50% thiol oxidation caused the oxidation of 0·7 mole SH/mole LAHPO. Further addition of LAHPO decreased the number of moles SH oxidized/mole LAHPO added. Furthermore, subsequent addition of $NADPH_2$ and glutathione reductase (EC.1.6.4.2) reversed the oxidation by 60%, suggesting that GSSG was the major reaction product. The amount of LAHPO needed to oxidize GSH and the proportion of GSSG formed suggest that the reaction products were GSSG (60%) and glutathione sulphonic acid (40%).

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The Inactivation of Xanthine Oxidase by Oxidizing Agents and *p*-Chloromercuribenzoate

By R. C. GREEN and P. J. O'BRIEN. (*Department of Medical Biochemistry, Birmingham University*)

The inactivation of xanthine oxidase preparations by *p*-chloromercuribenzoate (pCMB) and oxidizing agents has been attributed to SH modification (Webb, 1966).

Using 10^{-7}M highly purified enzyme in the presence of 10^{-4}M-xanthine, 50% inactivation occurred after 15 min. anaerobic incubation with $2{\cdot}7 \times 10^{-5}$M-*o*-iodosobenzoate or $1{\cdot}8 \times 10^{-4}$M-H_2O_2 in tris buffer, pH 8·2, at 23°. No inactivation occurred in the absence of xanthine. The inactivation was proportional to the concentration of iodosobenzoate or H_2O_2, and was dependent on the time of incubation and the pH. Inactivation by H_2O_2 was 52% less at pH 6·2.

50% inactivation by iodosobenzoate or H_2O_2 in 1 min. could not be reversed by immediate addition of excess cysteine (up to 5×10^{-2}M). However, even after 95% inactivation with pCMB, 71% of the initial activity was restored with cysteine. Furthermore, if the pCMB-inactivated enzyme was then incubated with sufficient H_2O_2 to cause 95% inactivation of the native enzyme, 45% of initial activity could be restored with cysteine. Since pCMB partially protected the enzyme from H_2O_2 inactivation, pCMB & H_2O_2 may affect the same group. pCMB, iodosobenzoate, and H_2O_2 have been shown to interact specifically with SH groups in enzymes (Webb, 1966; Little & O'Brien, 1966).

$2{\cdot}3 \times 10^{-5}$M-pCMB gave 50% inactivation of 10^{-7}M enzyme after 15 min. The presence of xanthine made no apparent difference, but most of the inactivation occurred instantaneously. Inactivation by pCMB was directly proportional to pCMB concentration, and, maintaining the same molar ratio of pCMB to enzyme, was about 30% greater on diluting the enzyme from 4×10^{-8}M to 4×10^{-9}M.

It is puzzling that such a large molar excess of pCMB is needed, as only 1–2 SH groups react during the initial inactivation (Bergel & Bray, 1959). This was confirmed for this preparation. For a 50%-inhibited enzyme $V_{max.}$ was slightly decreased, but K_m was increased from 10^{-5}M to 4×10^{-5}M with xanthine as substrate.

Since iodosobenzoate was more effective than pCMB after 1hr. incubation, then iodosobenzoate may react more specifically with SH groups than does pCMB.

No inhibition occurred with linoleic acid or *tert.*-butyl hydroperoxides or with succinoyl peroxide; and neither haematin nor cytochrome *c* stimulated the H_2O_2 inactivation, in contrast with the results obtained for the inactivation of an SH enzyme (Little & O'Brien, 1966). This may suggest that SH groups are not involved, or that they react non-catalytically with H_2O_2 and are inaccessible to other peroxides.

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