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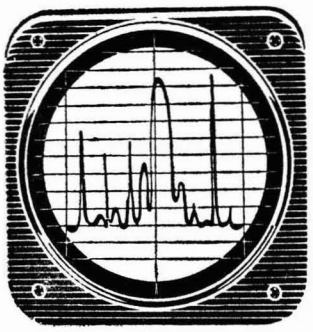

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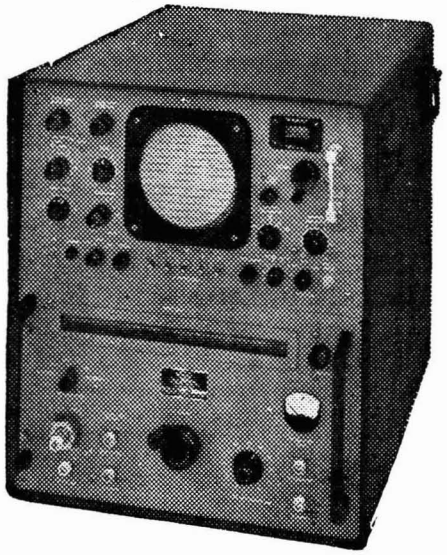

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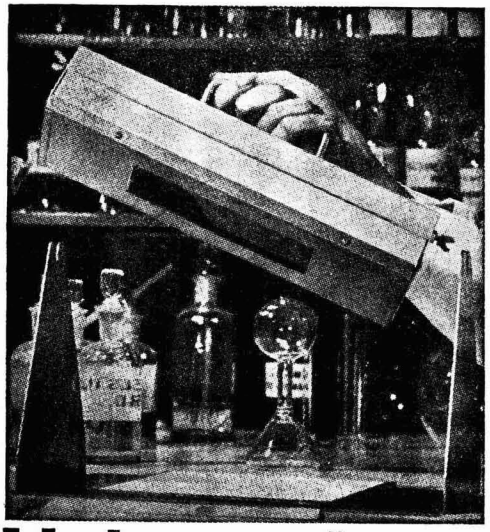

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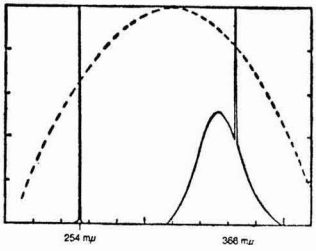

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Current Topics

Technical Know-how & Industry

ACQUISITION of technical know-how forms a vital clause in all collaborative agreements between indigenous industry and foreign industrial establishments. Invariably collaboration and know-how are offered and accepted as a package deal. It is not an uncommon experience that once foreign know-how is imported dependence on it continues indefinitely. The acquisition of foreign technical know-how has been and continues to be a subject of much debate and controversy. It was discussed at length at the meetings of the Standing Committee of the Central Advisory Council of Industries and the Joint Standing Committee for Scientific Research and Industry held recently in New Delhi. The aspect which received pointed attention at the meeting of the former committee was repetitive acquisition of foreign know-how, i.e. buying the same know-how again and again for each new industrial unit as and when it comes to be established. For obvious reasons this practice is wasteful and detrimental to national interests. Concern was voiced over India's continued dependence on foreign know-how.

The Standing Committee of the Central Advisory Council of Industries considered a number of measures to evolve a basis for importing foreign technical know-how. One of the measures considered was the creation of a single agency in the country for the acquisition of know-how for specific industries, particularly in those cases where there is not much possibility of the know-how becoming obsolete within a short time and also the number of units using the know-how is not large. This policy, implying in a broad sense the purchase of know-how once for all and passing it on to new units as and when they are established, though theoretically simple, presents several practical difficulties. For example, there are not many instances where know-how is identically repetitive. It is in the nature of scientific and technological inquiry that the process of improvement is a continuing one. Again, with a mixed industrial set-up in the country, it is doubtful whether all sectors of industry, particularly the private sector, would be a willing partner to an arrangement of this type. For a central agency to function as an efficient repository and supplier of know-how, it should have broader functions. Besides looking into the problem of obsolescence of know-how, it should have facilities for the solution of a variety of engineering problems in making available the know-how — indigenous and imported — to specific units interested in acquiring it, keeping in view their capacity, pattern of production, etc. In other words, it should have a corps of research workers charged with the responsibility of effecting continuous improvement in the stored know-how as well as a team of service technologists entrusted with the task of carrying the know-how to the factory and translating it to the commercial stage.

The meeting of the Joint Standing Committee for Scientific Research and Industry concerned itself mainly with collaboration between industry and scientific research organizations within the country for the exploitation of indigenously developed know-how. It was pointed out that though a good deal of industrial research has been and is being done in the country, the processes developed have not been utilized to a great extent. This inertia on the part of industry has to be overcome if Indian know-how is to find increasing acceptance. The factors responsible for this lack of interest on the part of industry to utilize indigenous know-how have to be thoroughly analysed and solutions found. One of the major stumbling-blocks in the way of making industry accept indigenous know-how is that Indian industry has no use for a process developed to the pilot plant stage and expects complete technical know-how which will enable it to undertake commercial production. Creating facilities on an adequate scale for the translation of research results to the commercial stage will involve much design and development engineering, industrial consultancy and allied work. Facilities now obtaining in the country for such work are meagre and steps have to be taken to establish these facilities.

Fifth Annual Convention of the Indian Chemical Society

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ABOUT 200 chemists from all over the country took part in the deliberations of the Fifth Annual Convention of the Indian Chemical Society, held at Roorkee during 3-5 November 1967. In all 96 papers, covering the major branches of chemistry, were presented and discussed. Besides, three special symposia, viz. (i) Magnetic resonance in inorganic compounds, (ii) Approaches to the study of surfactants, and (iii) Natural products, were organized. Nowadays, no spectroscopic technique, including IR spectroscopy, is more widely used by inorganic chemists working on the structures of complex inorganic compounds than the NMR spectroscopy. Comparatively a newer technique in the hands of inorganic chemists, it has helped immensely in the elucidation of structures and stereochemistry of many a complex inorganic compound. It was, therefore, natural that the symposium of magnetic resonance in inorganic compounds attracted the attention of a good number of researchers in the country, as is evident from the number of papers presented at the symposium. The idea of holding a symposium on the study of surfactants, which is actively pursued by the groups at Lucknow and Roorkee, was that those unfamiliar with the field may get a picture of the present position on a broad front. Very few papers were presented at the symposium on natural products which was arranged at a short notice.

Magnetic Resonance in Inorganic Compounds

In his inaugural address Dr C. R. Kanekar (Tata Institute of Fundamental Research, Bombay) surveyed the various areas in which NMR has made significant contributions to our knowledge of inorganic compounds. The usefulness of this method in the chemistry of (i) hydrido complexes, (ii) exchange and solution phenomena and (iii) reactions involving electron transfer was discussed. The data of the chemical shifts in Co(III) compounds were also discussed and their importance for understanding the structure of these compounds and for ascertaining the relative ligand field strengths of ligands were indicated.

Dr Monisha Bose (Saha Institute of Nuclear Physics, Calcutta) outlined the theoretical principles underlying NMR in paramagnetics as also the application aspect with particular reference to paramagnetic complex.

Dr R. Vijayaraghavan (Tata Institute of Fundamental Research, Bombay) presented his work on NMR as applied to solids and showed that how a study of line shapes, line-widths and line-shifts of the resonating nuclei gives an indication on the type of molecular motion, reorientation and structure. Some typical examples were also presented to illustrate these points. Magnetic resonance in paramagnetic substances was also briefly reviewed.

Dr A. Chakravorty (Indian Institute of Technology, Kanpur) presented a paper on nuclear resonance and some stereochemical problems. Two geometrical isomers of a compound, in general, differ with respect to the environment of nuclei. Thus *trans* $(Coen_2Cl_2)^+$ (en=ethylenediamine) shows only one NH_2 signal (H′ resonance) while the *cis*-complex shows two. The interaction of Co(III) amines with H_2SO_4 follows an interesting stereochemical path. The existence of *cis*- and *trans*-$[CO(CH_3OH)_4(H_2O)_2]^{2+}$ in solutions of $Co(ClO_4)_2$ in aqueous methanol was demonstrated. Adduct of TiF_4 and EtOH, i.e. $TiF_4.2EtOH$, below 0°C., shows two triplets (^{19}F) of equal intensity suggesting a *cis*-octahedral structure. However, at room temperature only a singlet is observed. This may be due to scrambling of fluorine atoms as a result of inter- and intramolecular exchange processes. Chelates of the type $M(A\text{-}B)_3$, where A-B is an unsymmetrical bidentate ligand, can exist in *cis* and *trans* forms. NMR proved to be extremely useful in diagnosing the geometry and in studies of *cis-trans* equilibria in such systems.

Dr U. R. K. Rao (Bhabha Atomic Research Centre, Trombay) and Dr J. Bacon and Dr R. J. Gillespie (McMaster University, Ontario, Canada) presented a paper on electric quadrupole relaxation of ^{51}V ($I=7/2$) in VOF_4^-. The temperature dependence study of the shapes of ^{51}V and ^{19}F NMR spectra of VOF_4^- in solution indicated that at a given temperature the spectrum of either nuclei in this species can be quantitatively explained in terms of the same spectral parameters, viz. coupling constant J and relaxation time T_1. It was also shown that at a given temperature the NMR spectrum of ^{51}V in this species shows more 'detail' than that of the corresponding ^{19}F. Some remarks regarding the electric field gradient at the vanadium site in this ion were made.

Prof. C. R. Kanekar and M. M. Dhingra (Tata Institute of Fundamental Research, Bombay) described investigations of isotropic proton shifts in pyridine-type bases complexed with diamagnetic nickel diethyldithiophosphate and nickel ethylxanthate. Isotropic contact shifts in the proton resonance spectra of pyridine-type adducts formed by Ni(II) diethyldithiophosphate and Ni(II) ethylxanthate were reported. The down field shift of all protons in pyridine was attributed to spin density in the σ-orbitals of the ligand. However, in α- and γ-picolines a high field shift for the CH_3 protons was observed. This was interpreted in terms of the spin density in the π-orbital of the ligand. It was found that the spin density in the π-orbital is higher in the adduct of the xanthate than in the dithiophosphate adduct indicating that it depends on the strength of the ligand fields in these complexes.

The application of ESR in the investigation of transition metal complexes, primarily with a view

to understanding the electronic and molecular structure of the complexes was discussed by Dr M. R. Das (Tata Institute of Fundamental Research, Bombay). The most useful quantities that are obtained from ESR spectra were g-values and ligand hyperfine splittings. How these experimentally determined quantities are made use of in understanding the spin-orbit coupling present in transition metal complexes and also in obtaining the covalency parameters for the metal-ligand bonds were discussed.

Finally, Prof. P. T. Narasimhan (Indian Institute of Technology, Kanpur) reviewed the application of nuclear quadrupole resonance spectroscopy to the study of inorganic compounds. ^{35}Cl and ^{37}Cl nuclei as well as Br and I nuclei are widely used as NQR probes. The NQR transition frequency is determined among other factors primarily by the local electric field gradient which in turn depends on the electronic structure. Townes and Dailey theory has been widely used to interpret the NQR data. π-Bonding in molecules can also be examined by NQR. A brief survey of the problems in organic chemistry that can be studied by NQR spectroscopy was also presented.

New Approaches to the Study of Surfactants

In the opening paper, Prof. A. C. Chatterji and Dewakar Prasad (Lucknow University) gave the mechanism of the detachment of soil particles from the substrate and explained how far micelles are responsible for soil detachment. A correlation between the critical micelle concentration (c.m.c.) with the detergent activity was made. It was reported that c.m.c. is not a sharp fixed concentration but a range of concentration in which micelles are completely formed. Within this range the size and the aggregation number of each micelle goes on changing till they attain the maximum values and beyond this range the change in aggregation number is only slow. The maximum concentration at which the single molecule in a solution of the detergent becomes constant and does not increase is given by surface tension measurements at its lowest value; on the other hand, the higher range at which the micelles are fully formed is found out by viscosity measurements. The density measurements in the case of non-ionic detergents give both the concentrations (lower and higher) of c.m.c. within which the micelles increase in size. Using the three types of the detergents, namely cationic, anionic and non-ionic, the following results were observed: (i) the c.m.c. is not an important factor in detergency; (ii) the maximum detergency occurs after the higher concentration range of the c.m.c. has already been attained; and (iii) this detergency value decreases slowly as the concentration is further increased.

Prof. W. U. Malik and A. K. Jain (University of Roorkee) described the use of electrometric and radiotracer techniques in the study of surfactants. A new approach to the study of surfactants involves the study of the effect of detergent ion concentration on the solubility of metal soap with the help of radiotracer technique. The effect of potassium laurate on the solubility of cobalt laurate in aqueous solution, employing ^{58}Co was reported. The solubility of cobalt laurate decreased with increasing potassium laurate concentration till the c.m.c. region was reached, while above c.m.c. the solubility remained almost constant.

Spectrophotometric and polarographic studies on surfactant-dye interaction were the subject of two more papers presented by Prof. Malik and coworkers. Polarographic reduction of Congo Red and Alizarin Red S in the presence of cationic surfactants and that of Methylene Blue in the presence of anionic surfactants, viz. dodecane sulphonic acid (DS) and dioctyl sodium sulphosuccinate (Manoxol OT), provided the following information:

(i) A gradual decrease in the limiting current is observed by the addition of increasing amount of surfactants resulting in the formation of precipitates of an insoluble dye-surfactant complex.

(ii) A linear behaviour is observed from the plots between the decrease in wave height and surfactant concentration.

(iii) When stoichiometric amounts or excess of the dye is present, a fine precipitate settles out slowly. Excess of the surfactant reduced the flocculation resulting in the formation of a stable suspension, non-reducible at the d.m.e.

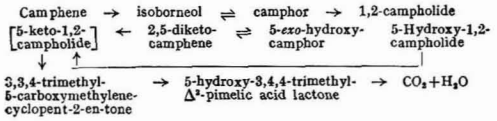
(iv) The binding ratio of Congo Red and Alizarin Red S with CTMAB and CPB, and that of Methylene Blue with Manoxol OT can also be computed from the polarographic data. In all the cases, the surfactant-bound dye ratio is found to be independent of the surfactant.

(v) In the interaction of Methylene Blue with DS, a second wave of constant height appears after the normal reduction wave in the presence of excess of DS. The existence of the second wave of constant height can be explained on the assumption that the dye-surfactant complex formed gets absorbed on the d.m.e. Moreover, a shift in half-wave potential towards the more positive side is observed.

Natural Products

Dr P. K. Bhattacharya and K. S. Khanchandani (Antibiotics Laboratories, Rishikesh) presented a paper on microorganisms as tools in the study of biogenesis of oxygenated terpenes. They explained that a soil pseudomonad capable of growing on camphene as the sole source of carbon was found to accumulate small amounts of isoborneol, camphor, camphenilone, 5-*exo*-hydroxycamphor, 1,2-campholide, 2,5-diketocamphene and 3,4,4-trimethyl-5-carboxymethylenecyclopent-2-en-1-one in the fermentation medium. Sequential adaptation studies with intact resting cells indicated that camphor or isoborneol grown cells grew on isoborneol or camphor without any time lag but growth on camphene took place after a lag period of 48-60 hr. Further, camphene-grown resting cells oxidized isoborneol, camphor, 5-*exo*-hydroxycamphor, 2,5-diketocamphene, 1,2-campholide, 5-*endo*-hydroxycamphor and 3,4,4-trimethyl-5-carboxymethylenecyclopent-2-en-1-one at comparable rates but were sluggish in oxidizing δ-lactone of 5-hydroxy-3,4,4-trimethyl-Δ^2-pimelic acid. Camphenilone, 2,3- and 2,6-diketocamphanes were not oxidized. Thus the

following metabolic pathway for camphene was formulated:



Dr Sukh Dev (National Chemical Laboratory, Poona) and Asha Chawla discussed the chemistry of resinous exudate from the trunk of *Ailanthus malabarica* De, which was found to contain several triterpenoids. Nine closely related compounds have been isolated from this source. The structures of the major component (malabaricol) and another minor component (epoxymalabaricol) were discussed.

Dr A. K. Barua and coworkers (Bose Institute, Calcutta) discussed the structure and streochemistry of lantanolic acid, a new triterpene from the leaves of *L. camara*.

Dielectric Properties of Solids*

G. SAROJINI
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EVERY solid has its characteristic dielectric behaviour depending on its structure, the type of lattice and the degree of internal molecular freedom. Studies on the properties of the dielectrics generally provide valuable information on a number of physical aspects, e.g. (i) the degree of polarization in an applied field; (ii) ionic or spontaneous polarization, if any; (iii) losses due to ionic movements, or dipolar orientation; (iv) the freedom of orientation of polar molecules; (v) relaxation times and relaxation mechanism; and (vi) formation of groups or other physical entities inside the solid and similar other features.

A literature survey of investigations on relaxation effects in dielectrics shows a large volume of work on solutions and liquids but little on solid dielectrics. A brief account on some aspects of the dielectric properties of solids and crystals is presented in this paper.

Theoretical Work

The dielectric behaviour of solids which are dependent on their structures is different from that of liquids. In solids, the dipoles would be more hindered and the restrictions in orientation of the polar molecules is usually caused by lattice forces[1] which cause decrease in dielectric constant and eliminate dielectric loss. In solids in the 'rotator phase', dipolar molecules have been found to possess sufficient orientational freedom to give dielectric constant comparable to those in the liquid state. Several of these molecules in the solid state were studied by Gutowsky and Pake[2] by means of NMR methods and effects of hindered molecular rotation about the axis of symmetry along which the molecular dipole lies were established. Rotation of the whole molecule or of intramolecular groups containing hydrogen was also observed by them. One of the first discussions on dielectric problems in solids was due to Debye who presented a 'two-site model' in which a molecular dipole could be aligned parallel or antiparallel to an external field. For no field, the two positions of equilibrium possessed by the dipole are equal in energy and opposite in direction separated by an energy barrier. Application of a constant field at the molecule lowers the energy of parallel alignment relative to the other and a small excess of dipoles will rotate into more favourable positions giving rise to polarization.

The magnitude of the energy barrier between equilibrium positions of the dipoles can be obtained from the temperature dependence of the frequency of maximum absorption[3] where the frequency factor A is constant

$$f_{max.}=(1/2\pi\tau)=Ae^{(-\Delta E/RT)} \qquad ...(1)$$

where ΔE is the energy difference between equilibrium positions, and τ is the relaxation time.

The two-position model was discussed in greater detail by Fröhlich[4], assuming expressions for transition probabilities of the form $(A_{21})_0=(A_{12})_0=(\omega_0/2\pi)\, e^{(-\Delta H/RT)}$ for no field. A treatment based on barriers of this kind was applied by Dryden and Meakins[5] to halide crystals with divalent cation impurities such as Ca, Ba and Sr. They estimated the lowest barrier height.

The existence of multiple relaxation times could be similarly interpreted on the basis of 'several-position model' by assuming a set of three or more stable sites and intervening potential barriers which are not all equivalent.

Experimental Work

The first experiments on solid organic compounds were conducted by Jackson[6]. He indicated the presence of the small ranges of relaxation times in ester — cetyl palmitate in nonpolar paraffin wax. The dielectric behaviour was accounted for in terms of the Debye theory. Further experiments were carried out by Sillars[7] and Pelmore[8] in a number of solid solutions of long chain esters in wax. The values of $f_{max.}$ from Eq. (1) showed linear variation

*Paper presented at the convention organized by the Physical Research Committee of the Council of Scientific & Industrial Research at the Banaras Hindu University, Varanasi, in March 1967.

with the molecular chain length of the ester and from this Fröhlich[9] developed a theoretical model and derived an equation for the relationship between the chain length and relaxation time for dipole orientation process. The magnitude of absorption and variation with temperature in solid solutions of long chain ketones and ethers were studied by Meakins[10] who obtained results in agreement with the Fröhlich theory. Several other workers[11-21] have done important work on the dielectric properties of solids. There are also a few excellent reviews[22-24].

Dielectric Studies on Crystals

The study of relaxation effects in crystals was mostly on crystals of the ionic type such as halides, alkaloids, etc. In ionic crystals one has to deal with electronic polarizabilities, induced moments from relative displacements of the ionic nuclei and their unbalanced electronic charge distributions. These motions are ineffective at optical frequencies and only electronic distortions contribute to the refractive index, but at low enough frequencies the ionic displacements give rise to much larger polarizabilities and dielectric constants of 5-30 or more for alkali halide crystals. Fröhlich[4], Szigeti[25] and Srivastava and Varshni[26] discussed the theory in detail. Brief reviews were given by Smyth[23] and Brown[27].

Work on single crystals at room and particularly at higher temperatures is relatively meagre. The dielectric properties of crystals provide information concerning the imperfection of the lattice, including colour centres. Dielectric measurements are also used to obtain the structures and interactions between molecules or ions in various materials. In crystals at ordinary temperatures, there are some unoccupied lattice sites. A vacant cation site is surrounded by anion and is, therefore, regarded as having a net negative charge. Conversely, a vacant anion site will have a net positive charge. A small proportion of vacancies of opposite sign are thus associated, forming dipoles which can change direction by the jumping of adjacent ions into vacancies. This forms the basis of a single dielectric absorption.

Built-in chemical impurities may carry an electric dipole moment, giving rise to dielectric losses. These are governed by a relaxation time which in turn is determined by a small activation energy. Colour centres which may be produced by exposing the specimens to ionizing radiation also make marked contribution to dielectric losses, especially at low temperatures. The losses due to impurities are deformation losses and those due to colour centres are colour centre dipole losses. The latter arise as electrons or holes and are trapped at various positions between which transitions can occur. Dielectric loss measurements are, therefore, interesting for the study of crystal structure.

Effect of Impurities

The effect of impurities was studied extensively by Dryden and Meakins[5], with particular emphasis on absorption in alkali halides when divalent impurities are present. They studied (i) the frequency at which the dielectric absorption occurs and (ii) the intensity of dielectric absorption at room temperature, at higher temperature (of the order of 130°C.) and after quenching the sample from 130° to 20°C.

When a divalent ion such as calcium replaces a positive ion in the lattice of an alkali halide, a positive ion vacancy is introduced. A dipole is formed if the cation impurity having an extra positive charge and the cation vacancy with an effective negative charge are associated near lattice sites. Dielectric absorption results from the rotation of this dipole. From the frequency measurements, the activation energy for the dipolar rotation has been determined. The number of divalent impurity cations associating with positive ion vacancies to form dipoles has been estimated from the intensity of absorption.

Another interesting study made by Meakins is the rate of aggregation of dipoles from the curves of the decay of intensity of absorption as a function of time following the quenching from 130° to 20°C. These experiments were carried out by them on a number of systems, Ca^{2+}+NaCl, KCl+Ba^{2+}, KCl+Sr^{2+}, NaCl+Mn^{2+}, etc. The last one is particularly important because ESR studies can be performed along with dielectric measurements. The intensity of dielectric absorption was also studied with respect to their dependence on concentration of divalent impurity. The results are given in Figs. 1 and 2. Fig. 1 shows the familiar Debye variation giving a relaxation time τ. The lowest

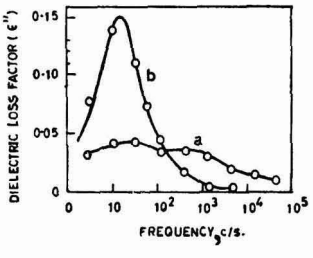

Fig. 1 — Dielectric absorption, measured at 20°C., as a function of frequency for NaCl containing $3{\cdot}6\times10^{-4}$ mole fraction of Ca^{2+} [(a) After storage for some time at 20°C.; and (b) immediately after quenching from 130° to 20°C.]

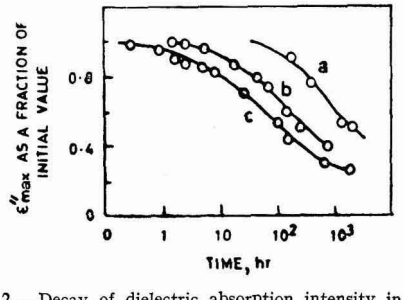

Fig. 2 — Decay of dielectric absorption intensity in NaCl (Ca) as a function of the time following quenching from 130° to 20°C. [Mole fraction of Ca^{2+}: a, $6{\cdot}8\times10^{-5}$; b, $2{\cdot}2\times10^{-4}$; and c, $3{\cdot}6\times10^{-4}$]

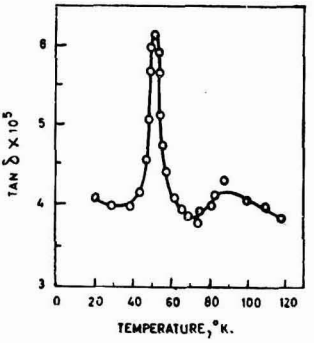

Fig. 3 — Dielectric constant of clear Brazilian quartz at 1 kc/s. as a function of temperature

curve, taken after storage for some time, shows the same value of relaxation time but decreased intensity of absorption. Association of dipoles into higher complex may, therefore, occur on standing at room temperature.

Fig. 2 shows the decay of dielectric absorption as function of time following quenching from 130° to 20°C. for three different concentrations. The curves are similar and from the shape of the curves Cook and Dryden[28] expected a third order reaction involved in the growth of complexes of higher order. The changes are interpreted in terms of reactions of the type: Ca^{2+}+free Na^{+} vacancy $\rightleftharpoons$ (Ca^{2+}—vacancy) dipole $\rightleftharpoons$ aggregates+some free vacancies or still higher complexes.

Effect of Irradiation

Volger and Stevels[29] have investigated the dielectric loss of different varieties of quartz including clear synthetic, smoky quartz, amethyst and others. A typical curve obtained by them between tan δ and τ is that given in Fig. 3. Such curves are also obtained in irradiated specimens and the results are interpreted in terms of natural imperfections in the crystals and those produced by irradiation. The investigation has shown that whereas migration losses predominate at normal and higher temperatures (i.e. due to migration of mobile ions), deformation losses predominate at low temperatures (i.e. losses which are related to defects in crystals). Another interesting result was that the quartz crystals have much smaller loss factors than glass.

Summary

The mechanism of dielectric loss in solid dielectrics, as interpreted by Debye and discussed in detail by Fröhlich, is briefly explained and some significant experimental results are described. The relaxation effects in crystals and how they arise from the presence of impurities and the effect of irradiation with particular reference to the published work on alkali halide crystals and quartz are also reviewed.

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Base-Catalysed Transformations of Olefins

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IN contrast to the abundance of literature on acid-catalysed conversions of olefins, base-catalysed transformations of olefins received, until recently, only scanty attention. Of late, activity is noticeable in this field and such transformations as (a) isomerization, (b) conjugation and aromatization of diolefins via dehydrogenation, (c) alkylation of aryl alkanes and (d) polymerization of mono-olefins induced by alkali metals and alkali metal compounds have been studied[1-3].

Catalysts used for these transformations include potassium hydroxide in alcohol[4,5], alkali alcoholates like sodium methoxide, potassium *tert*-alkoxides in the corresponding and also other alcohols[6], amides of sodium, potassium or calcium[7-10], potassium *tert*-butoxide in dimethylsulphoxide[11], high surface alkali metal dispersions on alumina[12-14], silica gel, sodium carbonate, etc., N-lithioethylenediamine complex[15] and sodium-organosodium compounds[16]. Most effective of all these catalysts are potassium *tert*-butoxide in dimethylsulphoxide and high surface alkali metals on activated alumina, the latter being effective even at, near or below room temperatures.

In the present context, it is proposed to review only (a) isomerization, (b) conjugation and aromatization (via dehydrogenation) of diolefins and (c) cyclization of medium ring trienes, laying emphasis on examples from terpene field.

Isomerization

Two general features of isomerization that are apparent are the migration of the double bond into a more substituted position and the favoured formation of *cis*-olefin over *trans*-olefin.

cis-2-Pentene (II) is formed preferentially from 1-pentene (I) (*cis*/*trans* ratio >10 at 1 per cent conversion) by the action of sodium-on-alumina. Carrying the reaction for 1 hr resulted in an equilibrium mixture containing 1-pentene (I; 1·6 per cent), *cis*-2-pentene (II; 26·4 per cent) and *trans*-2-pentene (III; 72 per cent)[13,14] (Chart 1).

Sodium-on-alumina isomerizes 1-butene (IV), *cis*-2-butene (V) or *trans*-2-butene (VI) into a similarly composed equilibrium mixture of the three olefins (IV-V) in 3, 26 and 71 per cent yield respectively.

A large number of simple and alkyl butenes[9,10,12-14,17] and pentenes[13-15,18] were studied for base-catalysed transformations.

N-Lithioethylenediamine isomerizes tetrahydroelemene[19] (VII) and dihydrogeraniol[19] (IX) smoothly into the internal olefin (VIII) and citronellol (X) respectively (Chart 2).

Although α-pinene (XII) is not affected by N-lithioethylenediamine, β-pinene (XI) is quantitatively converted into α-pinene (XII)[19].

Each of the three *p*-menthenes, viz. 1-, 2- and 3-*p*-menthenes (XIII, XIV and XV respectively), on isomerization with sodium-organosodium catalyst yielded similarly constituted mixture of 1-, 3- and $\Delta^{8(9)}$-*p*-menthenes[20] (XIII, XV and XVI respectively) (Chart 3).

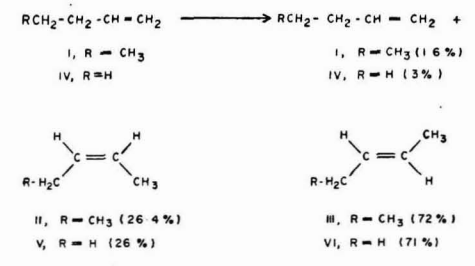


Chart 1 — Isomerization of 1-pentene and 1-butene

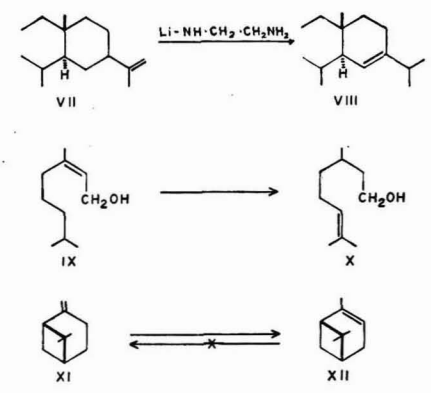


Chart 2 — Isomerization of tetrahydroelemene (VII), dehydrogeraniol (IX) and β-pinene (XII)

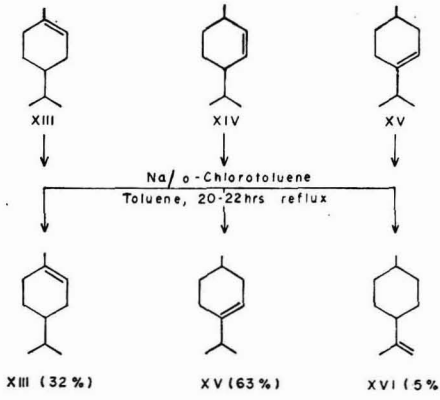


Chart 3 — Isomerization of *p*-menthenes

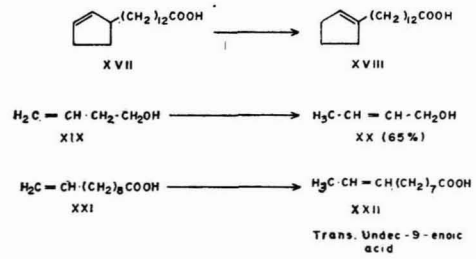


Chart 4 — Migration of a double bond into a more substituted position

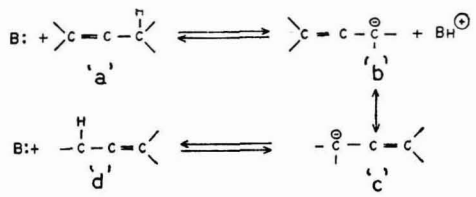


Chart 5 — Mechanism of isomerization

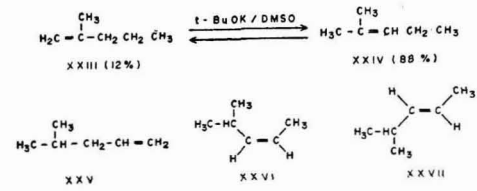


Chart 6 — Equilibration of 2-methylpentenes (XXIII and XXIV)

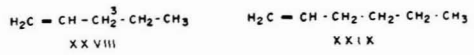
N-Lithioethylenediamine shifts the double bond into more substituted positions in chaulmoogric acid[21] (XVII), allylcarbinol[19] (XIX) and undecylenic acid[19] (XXI) (Chart 4).

Mechanism

The overall reaction can be represented as shown in Chart 5.

Starting either from 2-methyl-1-pentene (XXIII) or 2-methyl-2-pentene (XXIV), isomerization with potassium *tert*-butoxide in dimethylsulphoxide always results in isomeric mixture containing 12 per cent of the former and 88 per cent of the latter (Chart 6). No 4-methyl-1-pentene (XXV) or *cis*- and *trans*-4-methyl-2-pentenes (XXVI and XXVII) were formed, except on prolonged contact, indicating that the rate of isomerization past a tertiary carbon is much slower than that involving primary-secondary carbons, in conformity with the following known order of stability of carbanions: primary>secondary >tertiary.

The reaction can either be (i) intramolecular 1,3-proton shift or (ii) the anion can abstract a proton from (a) another olefin molecule or (b) solvent molecule. To distinguish between these alternatives Schriesheim *et al.*[11] investigated the isomerization of a 50: 50 mixture of pentene-1 (XXVIII) tagged with tritium at the allylic carbon atom and hexene-1 (XXIX) with potassium *tert*-butoxide in dimethylsulphoxide.



If it is an intramolecular 1,3-proton shift, the total radioactivity of tritium of the isomeric pentenes isolated after the reaction should be the same as that of pentene-1 (XXVIII), the starting material. On the other hand, if the carbanion ($a \rightleftharpoons b \longleftrightarrow c \rightleftharpoons d$) abstracts a proton from another olefin molecule, the tritium should appear on the untagged olefin (hexene isomers) after the reaction, or if the carbanion ($a \rightleftharpoons b \longleftrightarrow c \rightleftharpoons d$, Chart 5) abstracts a proton from the solvent molecule, the tritium activity of the tagged olefin (pentene-1) should decrease, the corresponding amount appearing on the solvent. These authors[11] demonstrated that hexenes, isolated after reaction, did not possess any tritium, 35 per cent of tritium appeared on the solvent and 65 per cent of the tritium was retained by the isomeric pentenes. Hence this reaction is 65 per cent intramolecular and the alternative (iib) operates to the extent of 35 per cent.

Cram and Uyeda[22] also arrived at similar conclusions from their studies of the isomerization (Chart 7) of optically active 3-phenyl-1-butene (XXX) with potassium *tert*-butoxide in *tert*-butanol-0-*d* at 75°.

Compound (XXX), isolated after the reaction, retained its total optical activity and was free of deuterium, while 3-phenyl-2-butene (XXXI) contained only 0·46 of one atom of deuterium per molecule, which was all present on C-1. Thus a minimum of 54 per cent rearrangement involved intramolecular proton transfer from C-3 to C-1.

Cram and Uyeda[6] also made detailed investigations on the mechanism of intramolecular hydrogen transfer in base-catalysed allylic rearrangements (Chart 8). The olefins chosen were (—)-3-phenyl-1-butene (XXX) and its 3-deuterio analogue in a variety of media containing deuterium donors (ROD) and protium donors (ROH) respectively, in the temperature range 25-145°. The extent of intramolecular 1,3-proton shift varied from 56 per cent for 3-phenyl-1-butene (XXX) in triethyl carbinol-0-*d*/potassium triethyl carboxide system to 6 per cent with 3-deuterio analogue in *tert*-butanol-tetramethylammonium hydroxide system. Other solvent systems studied were 1,2-dimethoxyethane-*tert*-butanol, *tert*-butanol-ethyleneglycol and their 0-deuterated analogues and also dimethylsulphoxide-methanol (91: 9 per cent). An isotopic effect of

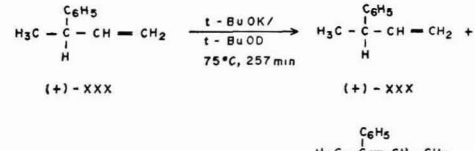


Chart 7 — Isomerization of (+)-3-phenyl-1-butene

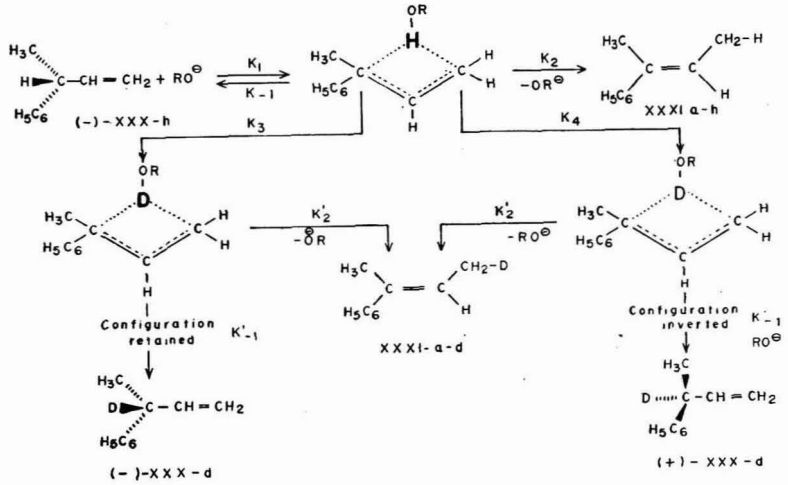


Chart 8 — Mechanism of base-catalysed isomerization of olefins

2·3-3·5 was observed. The reaction was fastest in potassium methoxide-dimethylsulphoxide-methanol system and slowest in ethylene glycol-potassium ethylene glycoxide system. *cis*- and *trans*-3-phenyl-2-butenes (XXXIa and XXXIb) are formed in the ratio of 5θ: 1. These and other data regarding isotopic exchange rates, when compared to rates of isomerization, led these authors[6] to postulate "allylic, ambident carbanions as intermediates, which are hydrogen bonded at both sites to the molecule of hydroxyl compound formed by abstraction of hydrogen or deuterium from starting material". The rates of collapse of this intermediate to rearranged olefin and isotopic exchange with solvent govern the extent of intramolecularity. The stereochemistry of exchange is controlled by whether isotopic exchange of this hydrogen bonded intermediate occurs at the front or back face of the ambident carbanion.

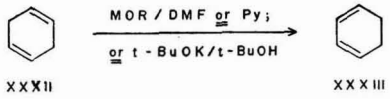


Chart 9 — Conjugation of 1,4-cyclohexadiene (XXXII)

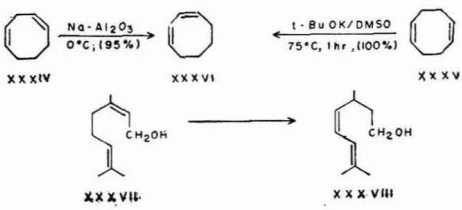


Chart 10 — Conjugation of cyclooctadienes (XXXIV and XXXV) and geraniol (XXXVII)

Stereoselectivity

Base-catalysed isomerizations are highly stereoselective and always the thermodynamically less stable *cis*-olefin is formed, in preference to the stable *trans*-olefin and hence must be kinetically controlled. This stereoselectivity is insensitive to changes in the base and solvent, in contrast to the rates of reaction. Schriesheim and coworkers[23] proposed that *cis*-allylic carbanion is thermodynamically more stable compared to the *trans*-anion and drew support from the parallel stabilities of *cis*- and *trans*-halopropenes.

Initially formed *cis*-olefins are convertible to *trans*-olefins under prolonged reaction conditions.

Conjugation and Aromatization

1,4-Cyclohexadiene (XXXII) is smoothly isomerized to give 1,3-cyclohexadiene (XXXIII) by alkali metal alkoxides in dimethylformamide or pyridine[24], without disproportionation (Chart 9).

The order of effectiveness of alkoxides, *t*-BuOK, (*t*-BuONa)>*i*-PrONa>EtONa>MeONa, is consistent with one of nucleophilicity of alkoxide anions and of solvation of cation.

1,4-(XXXIV) and 1,5-(XXXV) cyclooctadienes are converted into 1,3-cyclooctadiene (XXXVI) by sodium-alumina[14] and potassium *tert*-butoxide in dimethylsulphoxide[25] respectively (Chart 10).

Geraniol (XXXVII) was smoothly converted into the conjugated diene alcohol (XXXVIII) by N-lithioethylenediamine[26].

If the reaction of *d*-limonene (XXXIX) with sodium and promoters such as *o*-chlorotoluene, anthracene, etc., is stopped before completion, a mixture of dipentene (*dl*-XXXIX, 72 per cent), 2,4(8)-menthadiene (XL; 16 per cent), 3,8(9)-menthadiene (XLI; 4 per cent) and 10 per cent *p*-cymene (XLII) was obtained (Chart 11). No endocyclic diene was detected[27].

A facile transformation of Δ^3-carene (XLIII) by potassium *tert*-butoxide in dimethylsulphoxide into an equilibrium mixture of 60 per cent of (XLIII) and 40 per cent of Δ^2-carene (XLIV) was reported. N-Lithioethylenediamine gives either a mixture of Δ^3-(XLIII) and Δ^2-(XLIV) carenes or a mixture of (XLV) and (XLII) depending upon reaction conditions[28,29] (Chart 12).

β-Selinene (XLVI) underwent facile transformation (Chart 13), on reaction with N-lithioethylenediamine[20] into a heteroannular diene, (+)-δ-selinene (XLVII).

Other examples studied for conjugation include arylpropenes[30,31], 1,5- and 1,7-dienes[32], linoleic, linolenic and arachidonic acids[33,34], dihydro-[35] and hexahydronaphthalenes[36], indenes[37] and hexadienes[10].

Potassium *tert*-amyloxide in *tert*-amyl alcohol (184°C., 24 hr) equilibrates $\Delta^{1,8}$-(XLVIII) or $\Delta^{2,9}$-(XLIX) hexahydronaphthalenes to the same equilibrium mixture (Chart 14). Transoid dienes are more stable compared to cisoid dienes, with the same alkyl substitution[36].

Allenes

1,2-Cyclononadiene (LII) initially was isomerized to 1,3-diene (LIII) using potassium *tert*-butoxide in dimethylsulphoxide, but on prolonged treatment yielded an isomeric mixture, also obtainable starting from 1,5-cyclononadiene (LV), containing 6 per cent of 1,3-diene (LIII) and 94 per cent of 1,5-diene (LV). 1,3-Diene (LIII), although conjugated, is destabilized by the large interplanar angle imposed by ring strain (Chart 15).

1,2-Cyclodecadiene (LVI) was isomerized by potassium hydroxide in diethyl carbitol at 190°C. (48 hr) into 1,3-(LVII) and *cis*-, *trans*-1,4-(LVIII) cyclodecadienes[2].

N-Lithioethylenediamine aromatizes 4-vinylcyclohexene[15] (LIX) and 1,2,4-trivinylcyclohexene[38] (LXI) to ethylbenzene (LX) and 1,2,4-triethylbenzene (LXII) respectively (Chart 16).

Limonene[15,27,39] (XXXIX), phellandrene[15,39] (LXIII), sabinene[19] (LXIV), terpinolene[26] (LXV), perillyl alcohol[26] (LXVI) and carveol[26] (LXVIII), all give *p*-cymene (XLII) on treatment with bases under a variety of conditions (Chart 17).

β-Elemene[19] (LXX) and zingiberene[19] (LXXII) are likewise aromatized by N-lithioethylenediamine, the former losing the bulky ethyl group in the process (Chart 18).

N-Lithioethylenediamine aromatizes one of the rings in γ_1-cadinene[26] (LXXIV), γ-cadinene[19] [(LXXX) prepared from khusinol (LXXVIII)], khusol[19] (LXXVI), khusinol[19] (LXXVIII) and himachalenes[19] (LXXXI, α- and β-) (Chart 19).

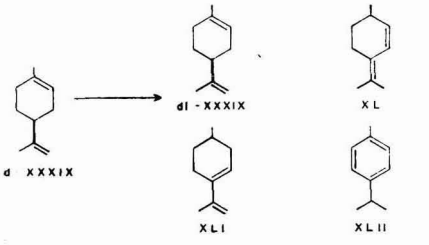


Chart 11 — Transformation of *d*-limonene (XXXIX)

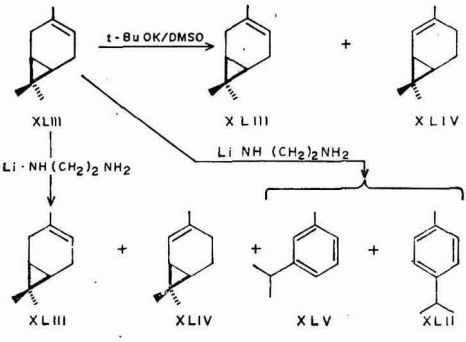


Chart 12 — Isomerizations of Δ^3-carene (XLIII)

Chart 13 — Isomerization of β-selinene (XLVI)

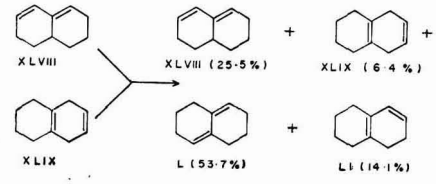


Chart 14 — Equilibration of hexahydronaphthalenes

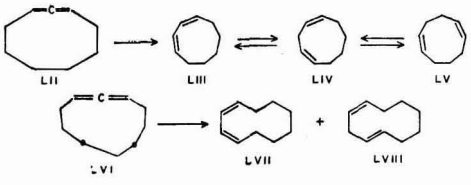


Chart 15 — Transformations of medium ring allenes

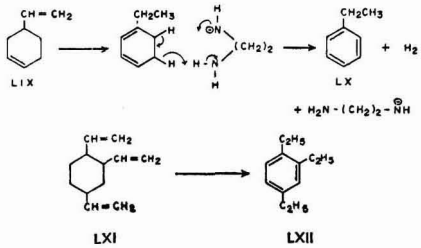


Chart 16 — Aromatization of 4-(LIX) and 1,2,4-tri-(LXI) vinylcyclohexenes

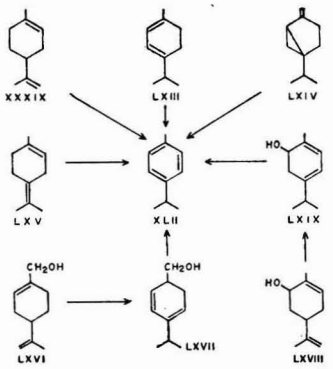


Chart 17 — Aromatization of limonene (XXXIX), phellandrene (LXIII), sabinene (LXIV), terpinolene (LXV), perillyl alcohol (LXVI) and carveol (LXVIII)

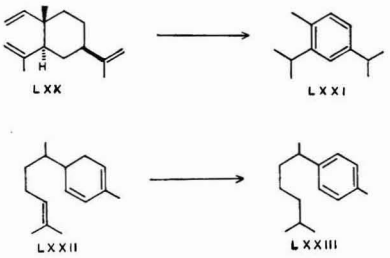


Chart 18 — Aromatization of β-elemene (LXX) and zingiberene (LXXII)

C-Ring of abietic acid (LXXXIII, R=H_2) and of 6-hydroxyabietic acid (LXXXIII, R=H, OH) is aromatized (Chart 20) to yield dehydroabietic acid (LXXXIV, R=H) in good yield by N-lithioethylenediamine[19].

6-Ketoabietic acid (LXXXIII, R=O) yields the corresponding phenolic acid (LXXXIV, R=OH) with the same reagent.

During the aromatization process, an alkyl group is lost in the cases of α-pyronene (LXXXV), 5,5-dimethyl-3-methylene cyclohexene (LXXXVII) and 6-ethyl-6-methyl-1,3-cyclohexadiene[16] (LXXXIX) (Chart 21).

Cyclization

1,2,6-Cyclononatriene (XCII) cyclizes under the influence of potassium *tert*-butoxide in dimethylsulphoxide (70°; 2 hr) to afford 4,7-dihydroindane (XCIII) and bicyclo[4.3.0]1,8- or 4,6-diene[25] (XCIVa or XCIVb) (Chart 22).

4,7-Dihydroindane (XCIII) precedes the bicyclo-(4.3.0)diene (XCIVa or XCIVb) in the reaction sequence. Gardener and coworkers[25] later identified one of the two short-lived intermediates, noticed to be present in an incomplete reaction product, as *cis*-bicyclo[4.3.0]nona-2,4-diene (XCVIII).

Watthey and Winstein[40] studied the base-catalysed transformation of *cis,cis,cis*-1,4,7-cyclononatriene (XCV) (Chart 23) by potassium *tert*-butoxide in dimethylsulphoxide (25-75°).

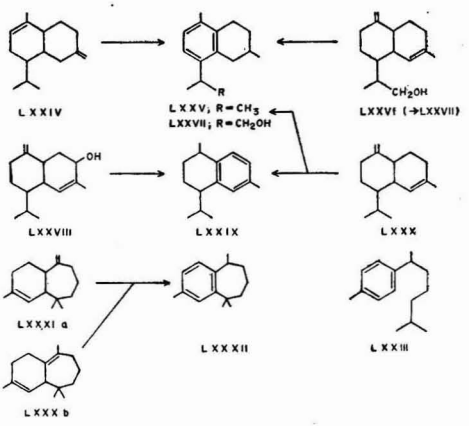


Chart 19 — Partial aromatization of γ_1-cadinene (LXXIV), γ-cadinene (LXXX), khusol (LXXVI), khusinol (LXXVIII) and α- and β-himachalenes (LXXXIII, a and b)

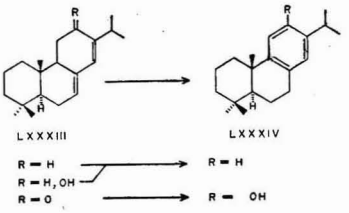


Chart 20 — Partial aromatization of abietic (LXXXIII, R = H_2), 6-hydroxyabietic (LXXXIII, R = H, OH) and 6-ketoabietic (LXXXIII, R = O) acids

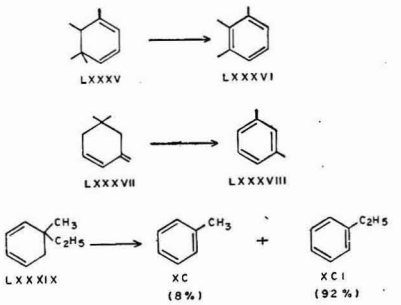


Chart 21 — Aromatization with concomitant dealkylation of geminally substituted cyclohexadienes

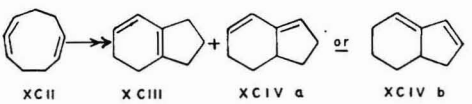


Chart 22 — Cyclization of 1,2,6-cyclononatriene

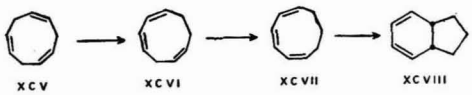
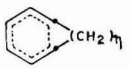


Chart 23 — Cyclization of 1,4,7-cyclononatriene

They suggested 1,3,6-triene (XCVI) to be the intermediate even in the transformation of 1,2,6-triene (XCII), which subsequently follows the same sequence as for 1,4,7-triene (XCV). The bicyclic homoannular diene (XCVIII) undergoes further isomerization to compounds (XCIII) and (XCIV) by way of at least one other unidentified isomer.

Under controlled reaction conditions[41] it was possible to isolate 1,3,6-triene (XCVI) and isomerize it further according to the above shown route. The transition state for the cyclization was proposed to be a 'homobenzene' (XCIX).



XCIX

Zerumbol[42] (C, R=H, OH) undergoes transannular cyclization and oxidation (Chart 24) on treatment with N-lithioethylenediamine. Zerumbone (C, R=O) under similar conditions was polymerized.

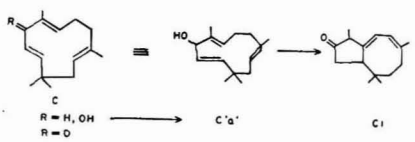


Chart 24 — Transformation of zerumbol

Intramolecular 1,5-Proton Shift

Cram and coworkers[43] recorded an interesting intramolecular 1,5-proton shift (Chart 25) in the compound (CII) which was converted into compound (CIII) by base. By carrying the transformation in deuterated solvent in the presence of different bases, 17-98 per cent intramolecularity was observed.

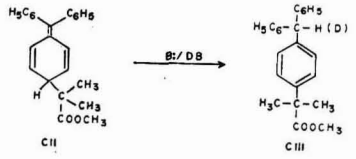


Chart 25 — Intramolecular 1,5-proton shift

Summary

Base-catalysed transformations such as isomerization, conjugation and aromatization (via dehydrogenation) of diolefins, and cyclization of medium ring trienes have been reviewed, citing examples mainly from the field of terpenoids. Mechanism and stereoselecting of the isomerization have been discussed. An interesting intramolecular 1,5-proton shift has also been recorded.

Acknowledgement

The author wishes to place on record his debt of gratitude and sincere thanks to Dr Sukh Dev for his kind encouragement during the preparation of this article, and his sincere thanks to Shri S. Venkataraman for typing the manuscript.

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Biosynthesis & Hydrolysis of Riboflavin & Flavin Coenzymes

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VITAMINS were discovered, chemically characterized and their role in nutrition established during the early decades of this century. Although attempts at establishing the effects of vitamins on animal physiology were successful, their biological role at the molecular level remained obscure.

The first major breakthrough in understanding the functions of vitamins was the discovery by Warburg and Christian[1] that riboflavin-5′-phosphate (FMN) was the coenzyme of 'old yellow enzyme' [reduced nicotinamide adenine dinucleotide phosphate (NADPH) dehydrogenase; EC 1.6.99.1]. This discovery was followed by the fractionation of the old yellow enzyme into a protein, 'apoenzyme' and the vitamin, 'coenzyme'. While the two fractions were inactive, the original enzyme activity was restored by mixing them[2]. This led to rapid advances towards the elucidation of the mechanism of action of enzymes, as well as the part played by the coenzymes in these processes.

In spite of these advances on the role of vitamins in enzyme reactions, our knowledge on the biosynthesis of riboflavin and its coenzymes was meagre until recently. This review attempts at an evaluation of the recent advances on the biosynthesis and hydrolysis of riboflavin, FMN and flavin adenine dinucleotide (FAD).

Biosynthesis of Riboflavin

Riboflavin is synthesized exclusively by plants and microorganisms. A few microorganisms like *Mycobacterium smegmatis* (bacterium), *Eremothecium ashbyii*, *Ashbya gossypii* and *Saccharomyces cerevisiae* BY_2 (yeasts) and *Aspergillus niger* (mold) excrete riboflavin into the culture medium. With the availability of chemically defined media for the growth of these organisms, the studies on the biosynthesis of riboflavin were initiated by MacLaren[3]. He studied the stimulation of riboflavin production by the inclusion of purines and pyrimidines in the growth medium of *E. ashbyii*. Adenine, guanine and xanthine stimulated the synthesis of riboflavin, while uracil showed a slight inhibition. None of these compounds had any effect on the growth of the organisms. These observations suggest that the purines stimulate the riboflavin synthesis, because they are the precursors of riboflavin or they are converted into its precursors. In the riboflavin excreting strain of yeast *S. cerevisiae* BY_2 (ref. 4), Giri and Krishnaswamy[5] observed the stimulation of riboflavin synthesis by various purines. Similar observations were made in the flavinogenic organisms, *A. gossypii* and *Candida flareri*[6].

Although the stimulation of riboflavin synthesis suggests the precursor activity of a compound, this observation cannot be considered conclusive. While urea stimulated riboflavin production in *C. flareri*[6], (^{14}C)-urea was not incorporated into the flavin molecule[7]. However, direct evidence for the precursor activity of purines came from the incorporation of radioactive purines and their precursors into riboflavin.

The uniformly labelled (U-^{14}C)-adenine was rapidly incorporated into riboflavin in *E. ashbyii*[8]. A degradative analysis of the radioactive riboflavin[7] revealed that the label was not only confined to rings B and C, but was also equally distributed between the carbons 2, 4, 4a and 9a of riboflavin (Fig. 1). However, the label from (8-^{14}C)-adenine was not incorporated into riboflavin[7]. These results suggest that after the removal of C_8, the purine residue was incorporated without further degradation. Later reports of McNutt[9,10] accounted for the source of nitrogens in riboflavin, when he showed the incorporation of (U-^{15}N)-xanthine and adenine into riboflavin. Paralleling the work of

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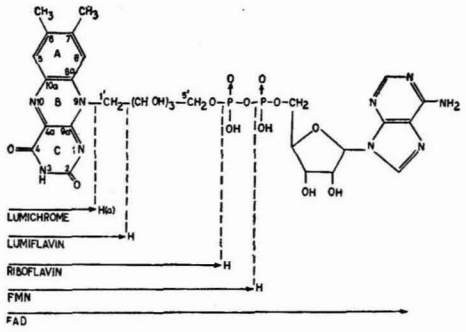


Fig. 1 — Structure of riboflavin, its coenzymes and alloxazine degradation products

McNutt[11], Plaut[12,13] studied the incorporation of various purine precursors into riboflavin. He found that the radioactive (^{14}C)-formate, $^{14}CO_2$, (1-^{14}C)- and (2-^{14}C)-glycine and (^{15}N)-glycine were readily incorporated into riboflavin in *A. gossypii*. The labelling pattern into purines and riboflavin was similar as shown in Fig. 2 (ref. 14).

Although purines minus their C_8 are the precursors of riboflavin, the actual nature of this purine derivative is still uncertain. The probable compounds are 4,5-diaminouracil or one of its derivatives. 4,5-Diaminouracil (I) was detected in the cultures of *E. ashbyii*[15], but the methods of its isolation do not preclude its existence *in vivo* as a ribose or ribitol derivative (Fig. 3). In riboflavin stimulation experiments, the purine nucleosides were no better than the purines for flavinogenesis[16,17]. These observations suggest that the purine nucleosides are not directly involved in flavinogenesis. Further, the ribitol residue of riboflavin does not arise from the ribose residue of guanosine. However, Goodwin and Horton[18] pointed out that in the presence of nucleoside pyrophosphorylase, guanosine could be in equilibrium with guanine and ribose-1-phosphate (1)

$$\text{Guanosine} + PO_4^{3-} \rightleftharpoons \text{guanine} + \text{ribose-1-}PO_4 \quad ...(1)$$

and that if the pool size of ribose-1-phosphate were much larger than that of guanine, then the guanine residue will be incorporated more than the ribose residue.

The plausibility of the formation of ribityl purine by the combination of ribityl derivative and a purine is indicated by the existence of CDP-ribitol[19] and the isolation of a partially purified enzyme forming CDP-ribitol[20]. Plaut[21] has suggested a possible mechanism for the formation of ribityl purine.

$$\text{Ribulose-5-}PO_4 + NADH \rightleftharpoons \text{ribitol-5-}PO_4 + NAD \quad ...(2)$$

$$\text{Ribulose} + NADH \rightleftharpoons \text{ribitol} + NAD \quad ...(3)$$

$$\text{Ribitol-5-}PO_4 + CTP \rightleftharpoons \text{CDP-ribitol} + PPi \quad ...(4)$$

$$\text{Purine} + \text{CDP-ribitol} \rightleftharpoons \text{9-ribityl purine} + CDP \quad ...(5)$$

$$\text{Purine} + \text{ribitol-5-}PO_4 \rightleftharpoons \text{9(5-}PO_4\text{-ribityl)-purine} \quad ...(6)$$

Although CDP-ribitol suggests itself as a good ribitol donor, there is no experimental support for its involvement in the biosynthesis of riboflavin.

Based on the incorporation of (1-^{14}C)-, (2-^{14}C)- and (6-^{14}C)-glucose into the various carbon atoms of ribitol moiety of riboflavin, Plaut and Broberg[22] suggest that ribitol is formed by at least two pathways of carbohydrate metabolism, the hexose monophosphate shunt and the transaldolase-transketolase system. It is not certain when or how ribitol is attached to the N_9 of riboflavin. All we know is that it is present in 6,7-dimethyl-8-ribityl lumazine (lumazine IV), a known intermediate of riboflavin.

A compound related to 4,5-diaminopyrimidine, a purine minus C_8 could be the precursor of riboflavin. An examination of the structure of the two compounds (I and riboflavin) shows that many more atoms in riboflavin remain unaccounted for. The discovery of 6,7-dimethyl-8-ribityl lumazine in flavinogenic organisms, *E. ashbyii*[23-25] and *A. gossypii*[26], was an important advance in the understanding of the biosynthesis of riboflavin. Structurally, lumazine (IV) bears close resemblance to riboflavin (V) and is short by only four-carbon atoms. These features of this compound make it a strong candidate for the position of an intermediate in riboflavin biosynthesis. The enhancement of

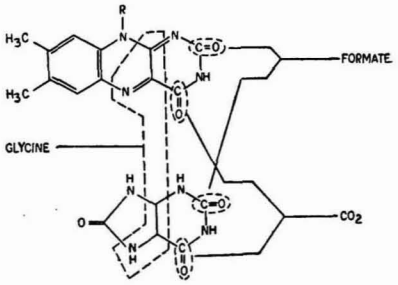


Fig. 2 — Labelling pattern into the riboflavin and purine molecules

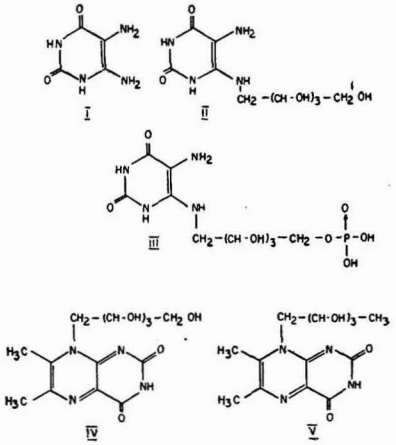
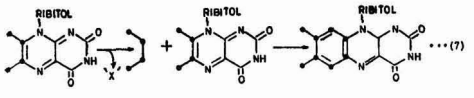


Fig. 3 — Precursors of riboflavin

riboflavin synthesis by the intact cells and the formation of riboflavin by cell-free extracts of *E. ashbyii* on the addition of lumazine clearly pointed to its precursor activity[27,28]. The direct proof for the precursor activity of lumazine was furnished by Maley and Plaut[29], who showed that the specific activity of riboflavin formed from (2-^{14}C)-6,7-dimethyl-8-ribityl lumazine was similar to that of this compound.

Several studies have been carried out to delineate the mode of formation of lumazine. 4-Ribityl-amino-5-aminouracil or one of its derivative smight be a precursor of lumazine. 4-Ribitylamino-5-aminouracil (II) and its phosphate ester (III) have been synthesized and found to be unstable. They were tested for precursor activity under a variety of conditions. Katagiri *et al.*[30] claim that the extracts of *E. ashbyii* and *Aerobacter aerogenes* form lumazine from II or III and acetoin (CH_3CHOH-CO-CH_3). However, Goodwin and Horton[18] could not achieve the conversion of these compounds either to lumazine or to riboflavin with the extracts of *C. flareri* under a variety of experimental conditions. Plaut[31] suggests that the enzyme preparation of Katagiri *et al.*[30] converts acetoin into butanedione, which then condenses with ribityl-aminouracil to form lumazine non-enzymically. Although such a reaction is possible, no unequivocal demonstration of the mode of formation of lumazine is available.

As already mentioned, the addition of four-carbon atoms to lumazine will complete the ring A of riboflavin. On the basis of the non-enzymic reaction, Masuda[23] suggested that acetoin might be the source of carbon atoms of ring A of riboflavin. However, the addition of acetoin to extracts of *Clostridium acetobutylicum* and *Escherichia coli ncapolitanus*[30] did not enhance the synthesis of riboflavin. Soon it became clear that riboflavin was synthesized as effectively by a 100-fold purified extract of *A. gossypii*[31] as by the crude extract using lumazine as the only substrate. Moreover, the activity of the extracts was unaffected by the addition of several four-carbon precursors, such as acetoin, diacetyl, DL-β-hydroxybutyrate, pyruvate, acetate, acetylphosphate, etc. Lastly, the failure to incorporate any radioactivity from (^{14}C)-acetate and (^{14}C)-glucose using partially purified enzyme and lumazine pointed to the absence of a separate four-carbon donor. This failure suggested that one molecule of lumazine donated the four carbons to a second molecule of lumazine to form riboflavin. This contention was proved by Plaut[31], when he found that for every molecule of riboflavin formed, nearly 2·3 molecules of lumazine disappeared. Further, Plaut[31] and Goodwin and Horton[18], using lumazine labelled with (^{14}C) in C_6,C_7 and the methyl groups attached to them, found that the four labelled carbons of one lumazine molecule are transferred to a second lumazine molecule, with the result that the specific molar activity of riboflavin is twice that of lumazine[32].



Riboflavin synthetase was shown to be present in extracts of plants[33] and flavinogenic microorganisms[30,34,35]; it was partially purified from extracts of baker's yeast, *Esch. coli*, *A. gossypii*[36] and spinach leaves[37]. The enzyme was absent in riboflavin-requiring organisms like *Lactobacillus casei* and *Enterococcus* IG AU[36]. The stoichiometry of the reaction was determined. The compound 'X', which is unstable was trapped by L-glyoxal or extracted into dilute mineral acids, in which it was relatively stable. The structure of this compound was elucidated[38] to be 4-ribityl-amino-5-aminouracil (II). Winestock *et al.*[39] studied the substrate specificity of the riboflavin synthetase from *A. gossypii*. Of the 29 lumazine analogues tested, only 6,7-dimethyl-8-(5′-deoxy-D-ribityl) lumazine (V) was slowly converted to 5′-deoxy-riboflavin. It is not surprising that 8-ribityl lumazines bearing substituents other than methyl groups at C_6 and C_7 are inactive. But the inactivity of epimeric tetrahydroxypentyl analogues of lumazine indicated the high degree of specificity of the enzyme. Several of the lumazine and riboflavin analogues exhibited competitive inhibition and the most potent inhibitor was 6,7-dihydroxy lumazine.

Recently, Harvey and Plaut[40] have obtained a highly purified riboflavin synthetase from baker's yeast extracts. They studied the kinetics and substrate binding properties of the enzyme. Reaction (7) is indicated to follow second order kinetics. However, the rate of reaction was found to be of either zero or first order kinetics, suggesting widely different affinities for the binding of the two molecules of lumazine to the enzyme. The enzyme forms isolatable complex with one molecule of lumazine. The binding with the second lumazine molecule was shown by the occurrence of an increase in the polarization of fluorescence. They proposed a model [Eq. (8)] for the binding and interaction of lumazines on the surface of the enzyme.

$$E+L \overset{K_1}{\rightleftharpoons} EL^d+L \overset{K_2}{\rightleftharpoons} EL^dL^a \overset{K_3}{\rightarrow} E+\text{products} \quad ...(8)$$

where E is the enzyme; L, lumazine; K_1, K_2 and K_3, rate constants of reactions; and the superscripts d and a represent the donor and the acceptor respectively. The enzyme binds lumazine and riboflavin analogues also. 6,7-Dimethyl-(5′-deoxyribityl)-lumazine (V) binds to the enzyme and is transformed into deoxyriboflavin. However, deoxylumazine is a good donor and a poor acceptor. When the labelled deoxylumazine-enzyme complex was reacted with cold lumazine, all the label was recovered in riboflavin and none in deoxyriboflavin, suggesting that the first lumazine molecule bound acted as a donor. The probable path of biosynthesis of riboflavin is shown in Fig. 4 (ref. 41). Reaction (7) releases a molecule of 4-ribityl-amino-5-aminouracil as a product. However, this compound does not accumulate in the flavinogenic organisms, suggesting that it is immediately utilized in the formation of a molecule of lumazine.

The enzymatic demonstration that lumazine is converted into riboflavin does not preclude alternate pathways for the biosynthesis of riboflavin *in vivo*.

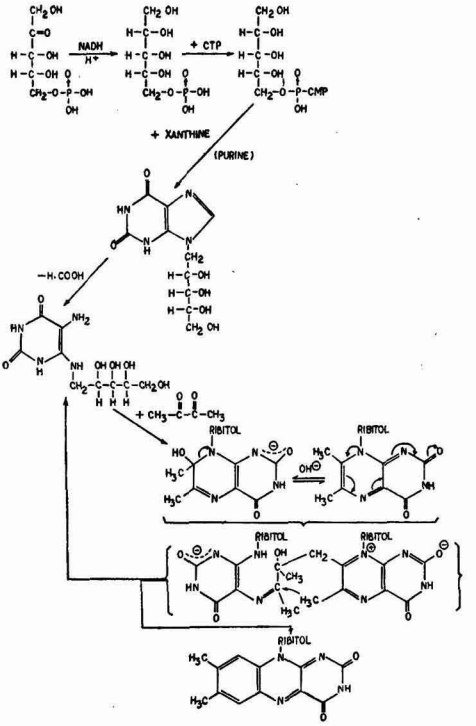


Fig. 4 — Proposed pathway for the biosynthesis of riboflavin

There are reports[42] to suggest that FAD is formed first and then it is hydrolysed to riboflavin. This has analogy to the observation that the end product of biosynthetic pathway of folate is dihydrofolate and not folate. Krishnaswamy[43] found similar amounts of flavin coenzymes in both flavinogenic and non-flavinogenic yeasts. Cerletti *et al.*[25] have also made similar observations about *A. gossypii*. In flavinogenic organisms there was a large excess of lumazine and riboflavin. As there was no increase in the amounts of FAD at any time, they suggested that riboflavin synthesis may not be taking place at FAD level.

Biosynthesis of Riboflavin Glycosides

Biosynthesis of riboflavin glucoside was first observed by Whitby[44] on incubating riboflavin and maltose with rat liver acetone powder. The biosynthesis of riboflavin glycosides has been demonstrated in *Aspergillus oryzae*[45], *Esch. coli*[46] and acetone-butanol producing bacteria[47]. The occurrence of this enzyme in seed extracts was demonstrated[48]. In plant seed extracts, the substrate activity of a number of sugars was observed, suggesting a fairly wide specificity. However, the reaction was slow and needed incubation periods of about 18 hr.

The enzyme from fish liver was found to be very active[49]. The enzyme from *Wallago attu* fish livers was purified 1000-fold (P. A. Kumar & N. Appaji Rao, unpublished results). The characteristics of the enzyme were found to be: *p*H, 7·5; temperature, 45°; and K_m for riboflavin and maltose $2\times10^{-3}M$ and $1\times10^{-3}M$ respectively. Maltose at higher concentrations inhibited the enzyme activity and K_i was found to be $8{\cdot}5\times10^{-2}M$. Other sugars were inactive. Heavy metal ions, such as Cu^{2+}, Hg^{2+} inhibited the enzyme, while ferric ion activated it.

The reaction products have been isolated by partition between phenol and water, cellulose column chromatography and finally by preparative circular paper chromatography. The pure compound was crystallized from 80 per cent aqueous ethanol. The properties of the crystalline product, flavin I, which was the major product of the enzyme reaction, were studied. Fluorescence of the flavin at various *p*H values and its absorption spectrum were similar to those of riboflavin. These results indicated that the flavin contained the isoalloxazine chromaphore. The flavin was at least 40 times more soluble in water than riboflavin. This was also reflected in the partition ratio of the new flavin between benzyl alcohol and water. The flavin on hydrolysis gave rise to one molar equivalent of glucose. Periodate oxidation also indicated that a glucose was in a glycosidic linkage with the terminal hydroxyl of the ribitol moiety of riboflavin. However, both α- and β-glucosidases failed to cleave off the glucose. This pointed to the existence of a different type of linkage in the compound. The exact chemical structure remains to be established.

Biosynthesis of FMN

Biosynthesis in higher plants — Although the occurrence of riboflavin and its nucleotides in plants was reported, no information was available on the quantitative distribution and biosynthesis of flavin nucleotides. Appaji Rao *et al.*[50] found that the major flavin in all the seeds analysed was FAD, with small amounts (about 5 per cent) of FMN and no riboflavin. The major flavin in the plumule was FMN, and that in the radicle, FAD. The plumule, the leafy bud, develops into the shoot system. FMN, which is involved in photosynthesis, appears to be readily available for the photosynthetic activity of the future shoot system. The radicle being the most actively respiring and growing part of the seedling requires FAD for the oxidative metabolism. The seedling from which the radicle was severed was unable to synthesize the flavins, suggesting that the radicle may be the site of synthesis of flavins. The content of flavin coenzymes and the enzymes synthesizing them increased during germination.

Giri *et al.*[51,52] showed the widespread occurrence of flavokinase (ATP: riboflavin-5′-phosphotransferase; EC 2.7.1.26) in higher plants. From the seeds of *Phaseolus radiatus* (green gram), a 75-fold purification of the flavokinase was achieved by the conventional methods of enzyme purification. Unlike yeast enzyme, the plant flavokinase had a very high temperature optimum (55°). While the enzyme was totally inactivated when incubated at 60°, much of the activity (90 per cent) was retained when it was incubated with its substrates, riboflavin and ATP. The substrates seemed to

protect the enzyme from heat inactivation. The *p*H optimum was 8·5. The substrate optimum for riboflavin was $1{\cdot}0\times10^{-4}M$ and for ATP $2{\cdot}0\times10^{-3}M$; the K_m values for the two substrates were $1{\cdot}5\times10^{-5}M$ and $1{\cdot}0\times10^{-4}M$ respectively.

The enzyme was specific to its substrates riboflavin and ATP. Neither lumiflavin nor lumichrome could replace riboflavin. While AMP was inactive, ADP could replace ATP. However, ADP was only half as active as ATP. Although Mg^{2+} activated the phosphorylation of riboflavin with both ADP and ATP, Zn^{2+}, which is an inhibitor of phosphatases and myokinase, inhibited the phosphorylation with ADP. These results suggest a possible contamination of myokinase, which forms a molecule of ATP from two molecules of ADP (reaction 9)

$$2ADP \longrightarrow ATP + AMP \qquad ...(9)$$

The fiavokinase reaction was subjected to the effect of various ions. While Mg^{2+} activated the reaction at all concentrations (up to $1\times10^{-2}M$), Mn^{2+} and Zn^{2+} inhibited it at higher concentrations. CN^-, MnO_4^-, Fe^{2+}, Cu^{2+} and Hg^{2+} were inhibitory. Li^+, Ca^{2+}, Cd^{2+} and Ni^{2+} had no effect.

Although the enzyme preparation did not hydrolyse FMN at *p*H 8·5 and temperature 55° (optimum conditions for FMN synthesis), it actively hydrolysed FMN at *p*H 4·3 and 49°. The hydrolytic activity might be due to a contaminating protein. These results suggest that the plant flavokinase reaction is essentially irreversible.

Biosynthesis in microorganisms — The first demonstration of enzymic phosphorylation of riboflavin was observed in brewer's yeast[53]. The enzyme was later extensively purified 500-fold[54]. The enzyme functioned optimally at *p*H 7·8-8·5 and temperature 38°. ADP was only half as active as ATP. The activity of ADP was on account of myokinase contamination even in the purest preparations of flavokinase. While ITP was inactive, AMP was a competitive inhibitor. There was absolute requirement for divalent cations, the most potent cation being Mg^{2+}. Although zinc could replace magnesium, it abolished the activity of ADP and the inhibition of AMP, suggesting myokinase contamination[55].

Substrate specificity studies for flavin were carried out by Kearney[56]. While dichloroflavin was effectively phosphorylated, arabityl flavin was phosphorylated slowly. Isoriboflavin [5,6-dimethyl 9(1′-D-ribityl) isoalloxazine], dulcityl, sorbityl, and galactoflavins were inactive.

The biosynthesis of FMN has been shown to take place in several microorganisms. Haley and Lambooy[57] found that of the 2-^{14}C-riboflavin administered to *Lactobacillus casei*, 87 per cent was incorporated into FAD, 6 per cent into FMN and 7 per cent was an unidentified product.

Krishnaswamy[43] studied the biosynthesis of FMN and FAD in *Saccharomyces cerevisiae* BY_1 and a mutant flavinogenic strain BY_2. The mutant strain showed diminished ability to synthesize the flavin coenzymes. Under conditions of inhibited flavinogenesis, the flavin coenzyme synthesizing activity of the mutant strain was restored to the normal. The flavokinase was purified 40-fold from the mutant strain. Like the yeast enzyme, it was optimally active at 37° and *p*H 7·8.

The flavokinase from *Lactobacillus arabinosus* was purified 40-fold by Snoswell[58]. The requirement for ATP as the phosphate donor was absolute. Mg^{2+} was the most effective divalent ion.

An enzyme for the phosphorylation of riboflavin distinct from flavokinase was reported by Katagiri and Imai[59]. Glucose-1-phosphate is the phosphate donor and the enzyme functions optimally at *p*H 5·4. However, no details of this work are available.

Biosynthesis in animals — According to Rudy[60], FMN is synthesized on incubating riboflavin and phosphate with intestinal phosphatase. A similar report by Hubner and Verzer[61] about the synthesis of FMN being effected by a reversal of phosphatase action could not be confirmed by Kearney and Englard[62]. It is now apparent that the reversal of the phosphatase action is an unlikely enzymological phenomenon on account of the energetics of the reaction. The synthesis of FMN is catalysed by a typical kinase 'flavokinase', with ATP as the phosphate donor. Only in the presence of ATP and Mg^{2+}, and at *p*H 7·4, the phosphorylation of riboflavin takes place in the intestinal mucosa[63] (reaction 10).

$$\text{Riboflavin} + ATP \xrightarrow{Mg^{2+}} \text{riboflavin-5′-phosphate} + ADP \qquad ...(10)$$

McCormick[64] studied this enzyme in rat liver system and found it to be localized in the supernatant fraction. The enzyme was initially purified to about 80-fold by the conventional methods of protein fractionation. Zn^{2+} was a more potent activator than Mg^{2+}. However, the enhanced activity of Zn^{2+} was due to its inhibitory action on phosphatases. With Mg^{2+} ions, the *p*H optimum was 7·5, while with Zn^{2+} it was 8·0. Among the trinucleotides, ATP was almost twice as active as GTP, which was the next best phosphate donor. The temperature optimum was 50°. The K_m value for ATP was $2{\cdot}0\times10^{-4}M$ and for riboflavin $1{\cdot}2\times10^{-5}M$.

Based on the substrate specificity of this enzyme for flavin molecule (described below), Arsenis and McCormick[65] prepared biochemically specific adsorbents for selective and extensive purification of flavokinase. Flavin-cellulose compounds were obtained by reacting 6-amino, and 7-amino-6,7-dimethyl derivatives of riboflavin with chlorocarbonyl methyl cellulose. The flavin-celluloses were used in a column chromatographic method for the purification of flavokinase. For this highly purified flavokinase, Zn^{2+} was no more active than Mg^{2+}, indicating complete removal of the contaminating phosphatases.

The specificity of the flavin molecule for the liver flavokinase has been studied extensively[64,66,67]. For all these studies the 80-fold purified enzyme was used. Analogues of riboflavin whose structure was altered in ring A, ring C and the ribityl side chain were tested for their substrate activity or for inhibition of riboflavin phosphorylation.

The essentiality of substitution at 6,7-position was indicated by the inactivity of desmethyl

riboflavin (9-D-ribityl isoalloxazine). The replacement of methyl groups in riboflavin by chlorine atoms had very little effect on the substrate activity. However, the activity of dibromo (6,7-dibromo [9-D-ribityl] isoalloxazine), 6-methyl, 7-fluoro and 6,7-dimethoxy flavins decreased in the order listed. The shifting of 7-methyl group to 5-position (5,6-dimethyl, [9-D-ribityl] isoalloxazine) as in isoriboflavin, totally abolished the substrate activity. This observation might suggest that position 5, which is close to N_{10}, must be unsubstituted for activity. Generally, the 6-substituted flavins were less effective than 6,7-substituted flavins as substrates.

Any alteration in ring C decreased the substrate activity. The substitution of 2 carbonyl oxygen with less electronegative groups, such as thio, imino, etc., decreased the substrate activity by favouring polarization towards a tautomeric form, which is not suitable for binding to the enzyme. Although 3-methyl FMN[68] is active as a coenzyme for several flavoproteins, it has no substrate activity for flavokinase.

There was great substrate specificity as regards the polyol side chain also. While D-xylityl, D-sorbityl, L-lyxityl and L-arabityl flavins were totally inactive, D-arabityl flavin was phosphorylated slowly. The lower and higher homologues of riboflavin, D-erythrityl and D-allityl flavins[69] as well as the 2′-deoxyribityl flavin were very poorly phosphorylated. The chain length is thus narrowly optimal for 5-carbon atoms and all the hydroxyl groups in D-configuration are essential for the activity. ω-Hydroxyl flavins with chain lengths of 3-6 carbons, flavins without primary alcohol group, flavins with very short side chains as lumiflavin and DL-glycerol were not phosphorylated, but were competitive inhibitors. The relative inhibitory potency of ω-hydroxyl flavins decreased very slightly with increasing chain length. This result suggests that the steric bulk of the side chain impedes to a very small extent the attachment of isoalloxazine moiety to flavokinase.

Thus, the substrate activity of the flavin depends on the structure of the flavin. However, there is no correlation between the oxidation-reduction potential of the flavin and its substrate activity. Taking into consideration the substrate activity of all the above flavins, the minimum requirements for the attachment of riboflavin to liver flavokinase have been proposed as shown in Fig. 5.

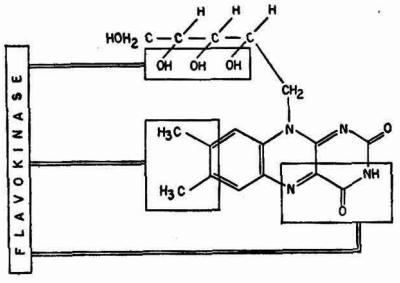


Fig. 5 — Sites of attachment of riboflavin to flavokinase

Biosynthesis of FAD

Biosynthesis in higher plants — In plants riboflavin is found usually in the form of its nucleotides. The increase in flavin content during germination clearly pointed to the ability of the plants to synthesize riboflavin and its nucleotides. However, there was no information available on the enzymatic mechanisms for the synthesis of flavin coenzymes.

The enzyme synthesizing FAD (ATP: FMN adenyl transferase; EC 2.7.7.2) is present in the seeds of a number of pulses[70]. From *P. radiatus*, the enzyme was purified 83-fold. It is optimally active at *p*H 7·5 and temperature 37°. The yeast and rat liver enzymes also are optimally active under these conditions. The K_m value for the substrate FMN is $4{\cdot}3\times10^{-5}M$ and for ATP $8{\cdot}0\times10^{-4}M$. While ADP and AMP did not replace ATP, riboflavin, isoriboflavin, lumiflavin and lumichrome did not replace FMN.

The inhibition of the enzyme by pCMB and arsenite and the reversal of this inhibition by monothiols like GSH, cysteine and mercaptoethanol, suggested the requirement of intact —SH groups for the activity of the enzyme. Mg^{2+} and Zn^{2+} activated the enzyme, while Mn^{2+} was inhibitory at $10^{-2}M$ concentration. Cu^{2+}, Ni^{2+}, Hg^{2+}, Ag^{+} and CN^{-} were inhibitory, while Co^{2+}, Fe^{3+} and Cd^{2+} had no effect.

In the presence of excess of pyrophosphate, yeast enzyme catalysed the reverse reaction leading to the formation of FMN. No such reverse reaction could be demonstrated with the plant and animal enzymes. However, in contrast to the widely different optimal conditions for the synthesis and hydrolysis of FMN, the optimal conditions (*p*H and temperature) for the synthesis and hydrolysis of FAD are the same. These results suggest that the action of FAD synthetase is reversible, whereas that of the flavokinase is essentially irreversible.

Biosynthesis in microorganisms — Klein and Kohn[71] claimed the synthesis of FAD by incubating human red blood cells with riboflavin. About the same time, Trufanov[72] reported the formation of FAD when riboflavin was incubated with rat tissue slices. He assumed that FAD was formed by a combination of riboflavin with ADP.

$$\text{Riboflavin}+\text{ADP}\longrightarrow\text{FAD} \qquad ...(11)$$

This mechanism of FAD formation looked unlikely when the mode of formation of NAD was determined.

$$\text{NMN}+\text{ATP}\rightleftharpoons\text{NAD}+\text{PPi} \qquad ...(12)$$

Schrecker and Kornberg[73], for the first time, isolated an enzyme synthesizing FAD from brewer's yeast. It was a pyrophosphorylase analogous to NAD synthetase.

$$\text{FMN}+\text{ATP}\rightleftharpoons\text{FAD}+\text{PPi} \qquad ...(13)$$

The enzyme was purified 91-fold. It was most active at *p*H 7·5 and showed a sharp optimum for Mg^{2+} ion concentration at $1{\cdot}5\times10^{-3}M$. ADP and metaphosphate and orthophosphate do not replace ATP and pyrophosphate respectively. Pyrophosphate, sodium fluoride (a pyrophosphatase inhibitor)

and large excess of NAD (which prevents the hydrolysis of FAD by virtue of its being the substrate for nucleotide pyrophosphatase) were included in the reaction mixture to demonstrate the reverse reaction[13].

Even the most purified enzyme contained high concentrations of nucleotide pyrophosphatase (dinucleotide: nucleotidohydrolase; EC 3.6.19) and inorganic pyrophosphatase. The flavokinase activity was also demonstrated under suitable conditions. In the presence of excess of riboflavin and increased amounts of enzyme, a restricted but definite synthesis of FAD was observed.

Giri and Krishnaswamy[74] detected FAD synthetase activity in *S. cerevisiae* and its flavinogenic mutant strain. The enzyme was optimally active at *p*H 7·5 and temperature 37°. A similar enzyme was observed in the extracts of *L. arabinosus*. As mentioned earlier, kinetic studies on the production of FAD and riboflavin in *C. guilliermondii*[42] suggest that riboflavin may be synthesized at the FAD level.

Biosynthesis in animals — Although the *in vivo* synthesis of FAD from riboflavin was well established, its synthesis in animal tissue homogenates could not be demonstrated. This was on account of the high hydrolytic activity of the homogenates. Deluca and Kaplan[75] found low FAD synthetic activity in mouse liver homogenates. Nevertheless, the soluble fraction obtained by centrifugation at 100,000 *g* was very active. The enzyme was present in rat liver, rabbit brain, beef liver and in different tissues of mouse. It was purified from rat liver. The final purification step involved gradient elution of the enzyme from a cellulose column.

The enzyme functioned optimally at *p*H 7·5. Mg^{2+} activated the enzyme up to a concentration of $1\times10^{-3}M$ and higher concentrations were inhibitory. Mn^{2+} partially replaced the requirement for Mg^{2+}. In the presence of cysteine, the enzyme was stable over an extended period of time. FMN and ATP were somewhat inhibitory at higher concentrations. Riboflavin could not substitute FMN, although it formed FAD slowly in the crude extracts. ADP was almost 70 per cent as active as ATP. However, the myokinase (ATP: AMP phosphotransferase; EC 2.7.4.3) contamination has not been ruled out. AMP was neither active nor inhibitory. The purified enzyme had no pyrophosphorylase activity and hence the reversibility of the reaction is not known.

Hydrolysis of Riboflavin and Flavin Coenzymes

Hydrolysis of riboflavin — Schopfer[76] reported the presence of lumichrome (6,7-dimethyl alloxazine) as well as riboflavin in the cell vacuoles of the upper epidermis of the bulb scales of *Allium* sp. The possibility of riboflavin undergoing enzymic hydrolysis was tested and a particulate enzyme was reported in a bacterium[77].

The degradation of riboflavin by an enzyme from *Crinum longifolium* bulbs has been demonstrated[78,79]. This involves the introduction of elements of water across a -C-N- bond of riboflavin to yield the intact heterocyclic ring, 6,7-dimethyl alloxazine and the pentitol, ribitol. This is analogous to the photodecomposition of riboflavin. The enzyme was purified 50-fold by the removal of inactive proteins by acid precipitation, $ZnSO_4$ precipitation and ethanol precipitation. The enzyme functioned maximally at *p*H 7·5, temperature 37° and with a substrate concentration of $1{\cdot}4\times10^{-4}M$ in the presence of $1{\cdot}0\times10^{-4}M$ of Mg^{2+} and $3{\cdot}0\times10^{-4}M$ of GSH (K_m value, $4{\cdot}7\times10^{-5}M$). Such a requirement of GSH and Mg^{2+} was not encountered in the bacterial enzyme[77]. The exact mechanism by which GSH activated the system is not known, but it might be due to its effect on the oxidation-reduction potential of the system or to the protection of -SH groups on the enzyme protein. While cysteine and ascorbic acid could replace GSH, others such as reduced NAD and NADP and Fe^{2+} could not. Among the divalent metal ions tested, only Mn^{2+} and Cd^{2+} partially replaced the requirement for Mg^{2+}. However, Li^+ activated the enzyme to a greater extent than Mg^{2+}. The effect of Li^+ was unique — the only instance where it has definitely been shown to activate an enzyme.

Among the metabolic inhibitors, NaF, NaCN, hydroxylamine, semicarbazide, benzylisothiocyanate and thiocyanate were inhibitory to varying extents. Using ascorbic acid in place of GSH in the reaction mixture, the inhibitory activity of pCMB was observed. This inhibition was reversed by the addition of thiol compounds like GSH, cySH, BAL or BME. This result suggested the requirement of -SH group(s) for the activity of the enzyme.

On studying the specificity of the enzyme, neither FAN, FAD nor isoriboflavin was found to be attacked by the enzyme. Thus, the blockade of the terminal alcohol group of riboflavin rendered the molecule unsuitable for enzymic hydrolysis. The site of substitution in ring A of riboflavin is also important, since isoriboflavin is not hydrolysed. Only FMN acted as a competitive inhibitor, suggesting that it had the right fit for the enzyme substrate (inhibitor) complex.

Hydrolysis of flavin coenzymes — Although the biosynthetic pathway for flavin nucleotide coenzymes is well understood[52,53,64,70], the pathways of their degradation had not been elucidated[80]. Kearney and Englard[53] suggested that FMN is hydrolysed by phosphatase action. Kornberg and Pricer[81] purified a nucleotide pyrophosphatase from potatoes which hydrolysed FAD. Several reports have appeared on the hydrolysis of the flavin coenzymes. FMN is cleaved very slowly to riboflavin and Pi, by a specific 5′-nucleotidase from bull seminal plasma[82], by an alkaline phosphatase[83] and also by ATPase[84] from cabbage leaves. FAD splitting enzymes have been found in rabbit muscle[85], seminal plasma[86], yeast[87] and tobacco roots[88]. However, there is no report about any detailed study on the enzymatic mechanisms of the hydrolysis of the flavin coenzymes. Appaji Rao *et al.*[89] and Kumar *et al.*[90] studied the hydrolysis of FMN and FAD respectively in detail.

FMN hydrolase — A number of plant seeds were screened for the occurrence of hydrolytic activity towards FMN. All of them showed this activity. Indeed to demonstrate the synthesis of the flavin

coenzymes, it was obligatory to incorporate into the reaction mixture an inhibitor of hydrolytic activity such as sodium fluoride.

The enzyme was enriched by about 200 times from the extracts of green gram (*Phaseolus radiatus*). The procedure entailed the precipitation of inert material by manganous sulphate, protamine sulphate treatment, fractional precipitation with alcohol and chromatography on CM-cellulose. During the purification, a gross portion of the non-specific acid phosphatase, inorganic pyrophosphatase, ATPase, 5′- and 3′-nucleotidases were removed. The enzyme preparations were tested for acid phosphatase activity with β-glycerophosphate as the substrate at various stages of purification. While in the crude enzyme extract the ratio of FMN hydrolase to acid glycerophosphatase activity was 1:5, in the purified preparation the ratio of the two activities is only 1:0·6. Thus, although the bulk of the non-specific phosphatase activity could be removed, the purified enzyme still exhibited some residual acid phosphatase activity. In this context, two possibilities are considered, viz. (i) the residual acid phosphatase activity is an inherent property of the FMN hydrolase activity or (ii) the acid phosphatase activity is due to a contaminating enzyme present in the purified preparation of FMN hydrolase. The greater heat lability of FMN hydrolase suggested the presence of an acid phosphatase as a contaminant. This is further supported by the difference in the behaviour of FMN hydrolase and glycerophosphatase activities in the presence of inhibiting ions. The effect of fluoride and Cu^{2+} ions on the two activities is particularly striking. Whereas fluoride at a final concentration of $1\times10^{-3}M$ caused 72 per cent inhibition of FMN hydrolase, only 9 per cent inhibition was observed in the acid phosphatase activity under the same conditions. The inability to remove the last traces of the acid phosphatase from the FMN hydrolase might be due to their closely similar physical properties.

FMN hydrolase was optimally active at 49° and *p*H 5·3. Maximum activity was obtained with FMN concentration of $4\times10^{-3}M$. The K_m value for this substrate was $1{\cdot}25\times10^{-3}M$. The enzyme was more active towards FMN than β-glycerophosphate, *p*-nitrophenyl phosphate, CMP, ribose 5′-phosphate, AMP and UMP in the decreasing order. The greater affinity of the enzyme (K_m value $1{\cdot}25\times10^{-3}M$) for FMN and the greater rate of reaction with FMN as substrate, suggested its identity as a specific FMN hydrolase. The entirely different conditions for optimal activity of FMN hydrolase (*p*H, 5·2; and temperature, 49°) from that of flavokinase (*p*H, 8·6; and temperature, 55°) suggest that the two enzyme activities were due to two different proteins. The purified enzyme of Heppel and Hilmoe[82] in contrast to this enzyme was highly active against cytidine 5′-phosphate and uridine 5′-phosphate, but showed only negligible hydrolysing activity towards FMN.

Phosphotransferase activity of FMN hydrolase — Generally, the biosynthesis of phosphorylated coenzymes is mediated by specific kinases requiring ATP[83]. Axelrod[91] showed that a certain phosphatase preparation has the phosphotransferase activity towards acceptors, such as simple alcohols and sugars, even in the absence of high energy phosphate donors. Brawerman and Chargoff[92] were the first to discover a group of enzymes capable of transferring esterified phosphoric acid to nucleosides, thereby effecting the synthesis of nucleotides. Recently, the phosphorylation of riboflavin to FMN by a phosphomonoesterase has also been reported[93].

Kumar and Vaidyanathan[94] observed that the FMN hydrolase purified from *P. radiatus* possesses phosphotransferase activity also. The enzyme was capable of transferring the cleaved phosphoryl moiety to suitable acceptors, such as simple alcohols, thiamine, pyridoxine, pyridoxamine and nucleosides, yielding the corresponding phosphate derivatives. It is interesting to note that the phosphorylation of pyridoxal and thiamine, which usually demand the participation of energy-rich pyrophosphate bonds (P∼P) like those of ATP, is effected by a simple hydrolytic enzyme involving a transfer of the cleaved phosphoryl moiety to the available acceptor.

The studies on the donor and acceptor specificity for the enzyme indicated that while both FMN and β-glycerophosphate were equally efficient as phosphate donors, there was wide disparity in the efficiency of the different acceptor compounds tested. The complete inactivity of pyridoxine and thiamine disulphide and the striking efficiency of thiamine, pyridoxamine and pyridoxal as acceptors suggested that the specificity pattern of the enzyme was so oriented as to favour the formation of active coenzymes like pyridoxamine and pyridoxal phosphates.

Both the FMN hydrolase and the phosphotransferase activities of the enzyme were unaffected by the sulphydryl reagent, pCMB. The optimum *p*H and temperature for both the activities were same (*p*H, 5·2; and temperature, 49°). Heating the enzyme at various temperatures inactivated both the activities. The effect of various ions towards both the activities was similar. The inhibition of fluoride and Cu^{2+} on the two enzyme activities was very similar. These results suggested that the phosphotransferase activity is an intrinsic and inherent function of FMN hydrolase.

Hydrolysis of FAD — FAD has been used to study the substrate specificities of a number of nucleotide pyrophosphatases. These studies did not exclude alternate mechanisms for the degradation of FAD or for the occurrence of specific enzymes hydrolysing FAD. A number of plant seeds examined showed hydrolytic activity against FAD[90]. The enzyme from *P. radiatus* was partially purified with ammonium sulphate and ethanol, and by negative adsorption on alumina C_γ gel. This enzyme was similar to the potato pyrophosphatase of Kornberg and Pricer[81], in that it catalyses the cleavage of the pyrophosphate bond in FAD and other nucleotides. The effect of substrate concentration, temperature, inhibition or activation by ions and substrate specificity of the two enzymes are very similar. However, some properties of this enzyme, i.e. stability, inhibition by cyanide and fluoride, etc., differ grossly from those of potato nucleotide pyrophosphatase. Potato nucleotide

pyrophosphatase is quite stable, whereas this enzyme is unstable at 0-5° and is inactivated by freezing and thawing. Cyanide and fluoride did not inhibit potato nucleotide pyrophosphatase, although both inhibited this enzyme.

To stabilize this enzyme, GSH was added during the purification of the enzyme. This altered the site of action of the enzyme. The reaction products were riboflavin, pyrophosphate and adenosine. The enzyme now cleaved the -C-O-P- instead of the -P-O-P- bonds. This change in the site of action of the enzyme due to pre-incubation with GSH was studied in detail. Both the GSH-treated enzyme and the untreated enzyme were optimally active at *p*H 7·2 and temperature 37°. The value of the Michaelis constant for FAD of both the enzymes was $1{\cdot}65\times10^{-5}M$. The preparation was free of flavokinase, FAD synthetase and FMN hydrolase, both in the presence and absence of GSH. Both the enzymes were specific to dinucleotides, such as FAD, NAD, NADP and ADP and inactive to thiamine pyrophosphate and mononucleotides, such as UMP, AMP and CMP. All these properties, i.e. *p*H, temperature affinity to the substrate and substrate specificity, suggest that the same protein was involved in the reaction.

The effect of sulphydryl reagents has indicated the alteration brought about in the enzyme protein. The untreated enzyme was inhibited by cyanide, sulphite, GSH and cysteine, while it was unaffected by pCMB and arsenite. On the other hand, the GSH-treated enzyme was inhibited by pCMB and arsenite, and unaffected by cyanide, sulphite and monothiol compounds. These results suggest that a disulphide (-S-S-) bond is essential for pyrophosphate (-P-O-P-) cleavage, whereas (-SH) sulphydryl group(s) are essential for the cleavage of phosphate ester (-C-O-P-) linkage. Moreover, the reversal of pCMB and arsenite inhibition by monothiols and dithiols respectively indicated the transformation of -S-S- of the protein to vicinal dithiol on GSH treatment.

Crystalline FAD hydrolase — Another enzyme hydrolysing FAD has been purified and crystallized from green gram seedlings[95]. From paper electrophoretic studies, the enzyme appears to be a homogeneous protein. This enzyme is at least 170 times more active than the partially purified enzyme of Kumar *et al.*[90]. The specific activity of this enzyme is 1333 mμ moles of FAD hydrolysed per min. at 37° in contrast to 7·5 mμ moles of the partially purified enzyme. The characteristics of this enzyme are also different: *p*H optimum, 9·4-9·6; temperature, 50°; and K_m, $6\times10^{-4}M$ of FAD.

The crystalline enzyme hydrolysed the pyrophosphate bonds (-P-O-P-). GSH at a concentration used in the partially purified enzyme showed inhibition, without transforming the enzyme to attack the phosphate ester bonds (-C-O-P-) of FAD. Similar inhibition was observed with mercaptoethanol.

The type of intramolecular change observed in the partially purified enzyme resulting in the change of the locus of attack is a novel phenomenon in enzyme chemistry. The unambiguous location of the changes in the molecular architecture would be essential to explain the mechanism of hydrolysis at the -P-O-P- and -C-O-P- bonds.

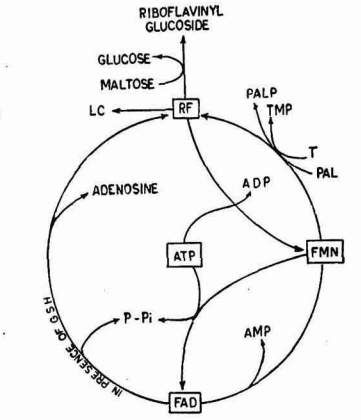


Fig. 6 — Riboflavin metabolism in plants

Summary

As detailed above, systematic studies have been carried out to elucidate the pathways for the biosynthesis and hydrolysis of flavin coenzymes and riboflavin. The studies in plants have led to a clearer understanding of the riboflavin metabolism. The results of these studies are summed up in Fig. 6.

Riboflavin was phosphorylated by flavokinase to FMN. FAD was formed by FAD synthetase utilizing FMN and ATP as substrates. FMN hydrolase not only dephosphorylates FMN but also shows transphosphorylase activity, leading to the formation of active coenzymes, such as pyridoxal phosphate. Normally FAD hydrolase showed pyrophosphatase activity, but on treatment with GSH, the enzyme altered its site of action to phosphate ester bonds. Riboflavin accepted glucosyl moieties from sugars to form the more soluble glycosides. Riboflavin was hydrolysed to lumiflavin by the action of an enzyme isolated from *Crinum longifoilum.*

The demonstration of these pathways permits us to speculate on the probable mechanisms controlling the availability of these nucleotides for the many reactions in which they participate. Our investigations cited in this review and that of Mitsuda[96] suggest that (1) binding of these coenzymes to proteins, (2) *p*H values at which these enzymes function optimally, (3) affinity of the substrates for the enzymes, (4) adenine nucleotides, pyrophosphate and reducing agents play a key role in the homeostasis.

It has been reported that flavoproteins do not act as substrates for hydrolytic enzymes[96]. Rao *et al.*[97] have shown that flavin bound to protein is available to reactivate apoflavoproteins and similarly Mahler[98] has shown that pyridine nucleotides bound to proteins are available for reaction.

The synthetic activities are optimal at neutral or slightly alkaline *p*H values. The hydrolysing

activities are present in leaves at acid *p*H values. Our results on the germinating seedlings of *Phaseolus radiatus* show that FMN hydrolysis is optimal at acid *p*H and seems to be different from that brought about by β-glycerophosphatase. It has in addition the interesting property of transferring the cleaved phosphate to pyridoxal, thiamine, etc., without the mediation of ATP, which is normally required for their biosynthesis. Regulatory significance of this observation remains to be assessed. The hydrolytic activities are also present at neutral as well as alkaline *p*H values. The affinity for their substrates is greater for the enzymes synthesizing these nucleotides than for the hydrolytic enzymes. All the synthetic reactions require ATP and the concentration of this nucleotide could regulate the synthesis. Pyrophosphate, a product of the FAD synthetase, Pi, a product of the FMN hydrolase, and AMP inhibit FAD and FMN hydrolysis. Naturally occurring reducing agents such as glutathione (GSH) and cysteine convert the nucleotide pyrophosphatase to a nucleotide phosphatase. The product of this reaction is pyrophosphate, which inhibits FAD hydrolysis at alkaline *p*H. NAD and NADP also inhibit FAD hydrolysis and this inhibition may be due to a competition for the catalytic site on the enzyme. The exact mechanisms of these inhibitions have to be worked out before assigning a regulatory role to any or all of these metabolites.

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Abbreviations

ADP	= 5′-(pyro)-adenosine diphosphate
AMP	= 5′-adenylic acid
ATP	= 5′-(pyro)-adenosine triphosphate
BAL	= 2,3-dimercapto-1-propanol
BME	= β-mercaptoethanol
CDP	= 5′-(pyro)-cytosine diphosphate
CM-cellulose	= carboxymethyl cellulose
CMP	= 5′-cytidylic acid
CTP	= 5′-(pyro)-cytosine triphosphate
Cys-SH	= cysteine
FAD	= flavin adenine dinucleotide
FMN	= riboflavin-5′-phosphate
GSH	= reduced glutathione
GTP	= 5′-(pyro)-guanosine triphosphate
LC	= lumichrome
Lumazine	= 6,7-dimethyl 8-(1′-D-ribityl) lumazine
NAD	= nicotinamide adenine dinucleotide
NADH	= reduced nicotinamide adenine dinucleotide
NADP	= nicotinamide adenine dinucleotide phosphate
NADPH	= nicotinamide adenine dinucleotide phosphate (reduced)
NMN	= nicotinamide mononucleotide
PAL	= pyridoxal
PALP	= pyridoxal phosphate
pCMB	= *para*-chloromercuribenzoate
Pi	= orthophosphate (inorganic)
PPi	= pyrophosphate (inorganic)
RF	= riboflavin
T	= thiamine
TMP	= thiamine monophosphate
UMP	= 5′-uridylic acid

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DNA-RNA Hybridization

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THE model of the hereditary material, deoxyribonucleic acid (DNA), proposed by Watson and Crick[1], was aimed at the elucidation of the mechanism of duplication of the genetic material and information transfer at the molecular level. The helical model based on base pairing has been subsequently shown to be correct in interpreting hereditary characters. The genetic information resides in the macromolecule in a coded form through the four bases, adenine (A), guanine (G), cytosine (C) and thymine (T). The specific base pairing between A and T and G and C is the clue to the replication of the double stranded structure.

In 1956, Volkin and Astrachan[2] of the Oak Ridge National Laboratory made a remarkable observation, which finally led to the elucidation of the mechanism of information transfer. When the bacterium *Escherichia coli* was infected with phage T2, a new type of RNA, distinct from the RNA already present in the host, was synthesized. The base composition of the former was similar to that of T2-DNA. This observation was first made by Volkin and Astrachan[2]; later similar observations were made in the case of other phages as well[3].

Spiegelman and his coworkers[4] could separate the newly formed RNA from the bulk of cellular RNA by density gradient centrifugation or by electrophoresis in starch column. Almost at the same time, Yčas and Vincent[5] reported the existence of a fraction of RNA in yeast, having a high rate of turnover and a composition very similar to that of yeast DNA, uridylic acid replacing thymidylic acid. Further light was thrown on the nature of this type of RNA by the studies of Brenner *et al.*[6] on T4-infected *Esch. coli*. These observations lent support to the Jacob and Monod concept of messenger RNA in protein synthesis[7]. According to this, the genetic message contained in the DNA molecule is transcribed through an intermediate messenger RNA (mRNA), which is translated at the ribosomal site. Numerous other observations supported this concept. For instance, Gros *et al.*[8] demonstrated the presence of DNA-like RNA by ^{32}P-pulse labelling experiments in the same way as was done by Brenner *et al.*[6] in the case of T4-infected *Esch. coli*.

Another set of observations employing a newly developed technique threw considerable light on the mechanism of information transfer from the genetic material. These were based on the demonstrations by Marmur and Lane[9] and Doty *et al.*[10] that native DNA, which is double stranded in structure, can be denatured by heating in salt solution, the result being the separation of two strands. DNAs from various sources could be characterized by the specific temperature (T_m) at which the transition from the native to the single stranded structure, referred to as melting, takes place. It was simultaneously observed that slow annealing leads to renaturation of the separated strands, whereas quick cooling keeps the DNA in the denatured state. It also became possible to demonstrate the formation of a variety of two- and three-stranded helical complexes[11-13] with synthetic ribopolynucleotides like poly A, poly U, etc. In search of a mechanism for the transformation of genetic message from DNA to RNA, Rich[14] thought of a novel experiment to demonstrate the formation of a hybrid helix containing both deoxyribose and ribose polynucleotides. For this, he used enzymatically prepared polyriboadenylic acid and the short chain poyfdeoxythymidylic acid chemically synthesized by Khorana and his coworkers[15]. The successful demonstration of the hybrid formation was the forerunner of the experiments designed by Hall and Spiegelman[16], which led to the development and exploitation of the widely used DNA-RNA hybridization technique.

The fact that T2-RNA possesses a base ratio analogous to that of T2-DNA suggested to Hall and Spiegelman[16] that the similarity may go further and extend to a detailed correspondence of base sequences. Therefore, purified T2-RNA was used for hybridization test with T2-DNA. Since the hybrid was expected to have a lower density than uncombined RNA, separation of the two was attempted by equilibrium centrifugation in CsCl gradients[17]. To ensure sensitive and unambiguous detection of hybrid, double labelling was used. The T2-RNA was marked with ^{32}P and T2-DNA with ^{3}H. These experiments demonstrated specific complex formation between the single stranded T2-DNA and the RNA synthesized subsequent to infection of *Esch. coli* with bacteriophage T2. No such hybrid formation was observed with heterologous DNA even if it had the same overall base composition as that of T2-DNA. It was concluded that T2-DNA and T2-specific RNA form a hybrid, because they possess complementary nucleotide sequences. These experiments were the first hybridization tests performed with naturally occurring DNA and RNA. Subsequently, Spiegelman *et al.*[18] demonstrated the presence of natural DNA-RNA hybrids in *Esch. coli* cells infected with T2, using the double labelling technique coupled with equilibrium centrifugation in CsCl density gradient in swinging bucket rotors. Konrad and Stent[19], however, could not detect the natural hybrid under conditions very similar to those employed by Spiegelman *et al.*[18]. In this connection it may be mentioned that Doerflir and his colleagues[20] first noted that RNA synthesized on a single stranded DNA template yielded a product markedly resistant to RNase, suggesting

*The author's training on DNA-RNA hybridization at the International Laboratory of Genetics and Biophysics, Naples, Italy, and personal discussions with Drs Spiegelman, Yankofsky, Gillespie and others have been of great help in the preparation of this review.

the appearance of a DNA-RNA hybrid in the reaction mixture. This was confirmed by Warnaar *et al.*[21], who characterized the density and thermal transitions of the product. Using the single stranded DNA of bacteriophage $\phi x174$ in similar studies, Chamberlin and Berg[22] and Sinsheimer and Lawrence[23] confirmed the conversion of the single stranded templates to DNA-RNA hybrid. Bassel *et al.*[24] also identified the RNase-resistant hybrid structure. Electronmicroscopic studies revealed the gradual conversion of the collapsed coil of the single stranded DNA template to a double stranded hybrid structure possessing circular morphology. In this connection an interesting observation made by Byrne *et al.*[25] is worth mentioning. They demonstrated electronmicroscopically the formation of a DNA-ribosome complex which was most probably formed through the hybridized complementary RNA on the template DNA.

As already mentioned, Gros *et al.*[8] demonstrated that normal *Esch. coli* cells synthesize the type of RNA (messenger) which had been detected in T2-infected cells. A similar conclusion was drawn by Hayashi and Spiegelman[26] by applying the hybridization test. For the isolation of the complementary RNA (so-called messenger), a step down culture technique was used. The cells were subjected to a shift from a rich to a synthetic medium containing ^{32}P. The isolated ^{32}P-RNA exhibited a base ratio analogous to its homologous DNA and was metabolically unstable. It was very heterogeneous in character, but possessed the ability to hybridize specially with its homologous DNA.

In the meantime, numerous reports from various laboratories (cf. reviews by Grunberg-Manago[27], Levintow[28] and Elson[29]) indicated the synthesis of mRNA (as conceived by Jacob and Monod[7]) on the specific DNA templates. The synthesis is catalysed by the widely studied and well-characterized enzyme RNA polymerase. It was also clearly established that the basic feature behind the synthesis of mRNA was the sequential arrangement of the ribonucleotides as dictated by the base sequences of the template DNA through base complementarity. The results of the DNA-RNA hybridization tests performed by Spiegelman and his coworkers[18,26] were in complete agreement with the results of *in vitro* studies. A dilemma was, however, faced regarding the origin of the other two classes of RNA in the cells, namely ribosomal (rRNA) and soluble RNA (sRNA). At least two distinct rRNAs (16S and 23S) were characterized and a large number of sRNA molecules were expected to occur in the cell. The *in vitro* studies were of very little help in establishing the mode of synthesis of rRNA and sRNA, though it was suspected that their origin may also be on DNA template. The final answer, however, came from the DNA-RNA hybridization tests performed by Spiegelman and his coworkers[30-34] and Goodman and Rich[35]. Yankofsky and Spiegelman[30] were the first to establish the existence of a sequence in *Esch. coli* DNA complementary to its rRNA, showing that a hybrid complex resistant to RNase is specifically formed with homologous DNA. The procedures developed provided a method of sufficient specificity and sensitivity to detect the hybridization even to the extent of 0·01 per cent of the DNA molecule. Further evidences were provided by Goodman and Rich[35] on the basis of saturation experiments, which suggested that the particular region of the DNA molecules involved in hybridization with rRNA corresponds to 0·1-0·2 per cent of the total genome. With the refinement of the technique, however, these values had to be modified[31]. Anyway, non-ribosomal RNA from the same organism was found not to compete for the same site. Subsequently, it was shown that the two classes of rRNA (16S and 23S) hybridize with the DNA genome at distinct sites and do not compete with each other for the same site. The implication of these results is discussed in detail later. Goodman and Rich[35] similarly demonstrated the existence of a sequence of nucleotides in DNA complementary to that of sRNA by forming RNase-resistant hybrid complexes between sRNA and DNA. Specific *Esch. coli* DNA-sRNA hybrids have also been made by Giacomoni and Spiegelman[34].

The original techniques introduced for the DNA-RNA hybridization tests have undergone extensive modifications. Very recently Milman *et al.*[36] attempted to study the structure of the DNA-RNA hybrids by X-ray diffraction analysis. Though the results obtained are preliminary in nature, extensive studies of the three-dimensional structure of the hybrid are expected to yield detailed information regarding enzymic duplication or transcription of the genetic material.

Techniques Employed in the Formation and Detection of DNA-RNA Hybrids

The technique originally used by Hall and Spiegelman[16] to demonstrate DNA-RNA hybrids involved incubation of denatured DNA and RNA in state of solution at high temperatures followed by cooling slowly down to room temperature (25-26°C.). Denatured DNA was prepared by heating native DNA (^3H-labelled) for 15 min. at 90°C. in 0·15*M* NaCl and 0·01*M* sodium citrate (*p*H 7·8) followed by quick cooling in ice. Hybridization with RNA (^{32}P-labelled) was conducted in 2×SSC (SSC= 0·15*M* NaCl+0·015*M* sodium citrate). The DNA-RNA hybrid formed was separated from unhybridized DNA and RNA by CsCl-density gradient centrifugation. The centrifugation was carried out in a swinging bucket rotor at 25°C. for 3-5 days. At the end of each run, the fractions were collected after piercing the bottom of the tubes. Samples were assayed for ultraviolet absorbing material and radioactivity. The nucleic acids in aliquots of the samples were precipitated with trichloroacetic acid, collected on a millipore filter and washed. The filter was air-dried and the radioactivity assayed in a liquid scintillation counter. Fractions containing both ^3H and ^{32}P indicated the presence of DNA-RNA hybrids.

Though the above-mentioned technique yielded quite convincing results, demonstrating the formation of DNA-RNA hybrids, it has several drawbacks. At higher temperature DNA-DNA hybrid formation also takes place. There may be mechanical trapping of small amounts of RNA in the DNA strands or partial hybridization due to accidental

coincidences of complementarity over small segments of DNA and RNA, which might vitiate the results. Hence, the technique was gradually modified and improved. Further, to achieve fruitful results, a few precautions had to be adopted. DNA had to be specially free of RNase, which was achieved by passing it through methylated serum albumin-kieselguhr column (MAK column). RNA contamination in DNA was removed by treating DNA with RNase (free from DNase). The RNA to be tested should be freed from other types of RNA by passing through a MAK column. In the case of sRNA and rRNA, however, the contamination by mRNA could not be avoided by this method. rRNA of high specific activity and free of significant contamination by labelled mRNA was prepared by subjecting a uniformly labelled culture to a chase in non-radioactive medium[30]. The technical difficulties inherent in using hybridization to establish the existence of complementarity between rRNA and DNA have been discussed in detail by Spiegelman[37]. An important property of a DNA-RNA hybrid is its resistance to ribonuclease digestion. This was first demonstrated by Schildkraut *et al.*[38] using synthetic polynucleotides. This property was utilized by Yankofsky and Spiegelman[30] to differentiate the true hybrid formed between rRNA and DNA from the non-specific (raw) hybrid. The complexes formed between rRNA and DNA of heterologous origin were completely sensitive to ribonuclease, whereas those involving homologous nucleic acids were resistant. The introduction of RNase treatment increased the sensitivity by removing RNA mechanically trapped in the strand of heat-denatured DNA and thus decreased the 'noise' and allowed differentiation between real and apparent or 'raw' hybrid. After RNase treatment even less than 0·1 per cent of complementarity can be detected with reliability.

The density gradient centrifugation originally used to isolate DNA-RNA hybrid (discussed earlier) involved lengthy centrifugation. Adopting a new medium for hybridization of DNA and RNA, Bautz and Hall[39] made a notable advance towards purifying RNA molecules having base sequence complementary to DNA. They used phosphocellulose acetate to immobilize denatured T4 phage DNA. The glucosylic hydroxyls of DNA would form covalent bond with the phosphate groups on the cellulose. DNA thus immobilized was found to form specific hybrids when incubated with RNA. Non-complementary RNA could then be washed away and complementary RNA reclaimed after decomposing the hydrogen bonds between DNA and RNA. The limitation of this column is that it can be used only for glucosylated DNA, which is present in some bacteriophages. Investigations of this reaction led to a method for immobilizing any high molecular weight DNA by physical entrapment in cellulose acetate gels or more readily in agar gels[40]. DNA thus immobilized can form hydrogen bonds with complementary molecules. The method of Bautz and Hall[39] was generalized by Bolton and McCarthy[40] who found that mechanical immobilization would suffice and developed agar columns containing denatured DNA trapped in the solidified gel. Preparation of cellulose acetate or agar in which DNA has been embedded has been described by Bolton and McCarthy[40]. Hybridization of RNA with such immobilized DNA can be done by incubating RNA (in 2×SSC) with DNA-cellulose acetate or DNA-agar either in slurry at 60°C. in a screw cap vial or in a chromatographic column heated by circulating water. The preparations heated in vials were then transferred to chromatographic columns for subsequent operations. The columns were washed with 2×SSC to remove the non-hybridized RNA. Recovery of the hybridized RNA was effected by washing with 0·01×SSC, which leads to the melting of the hybrid. Immobilization of DNA in cellulose acetate or agar column not only reduced the time of centrifugation, but also diminished the probability of DNA-DNA hybrid formation, which takes place when DNA and RNA are incubated in solution at higher temperatures.

Introduction of the DNA columns initiated the modification of the DNA-RNA hybridization technique based on immobilization of DNA. Britten[41] fixed DNA by ultraviolet irradiation to synthetic polymers, which were then employed as columns for the hybridization of RNA. This technique has, however, not been much used. In 1964, Nygaard and Hall[42] found that nitrocellulose filters strongly adsorb single stranded DNA as well as DNA-RNA hybrids, whereas RNA passes through the filters; this observation, besides making the hybridization technique more convenient, increased the capacity for handling a large number of samples. DNA-RNA complexes were formed by mixing RNA and DNA in solution at room temperature and then quickly shifting to a high temperature. Then the solution was filtered through the nitrocellulose membrane which adsorbed the DNA-RNA hybrid. The filter could be counted directly to measure the extent of hybridization. The technique of Nygaard and Hall[42] was modified by Gillespie and Spiegelman[43], and the modified technique is now by far the most convenient method for the quantitative assay of DNA-RNA hybrids. The procedure involves binding of denatured DNA to membrane filters, hybridizing RNA with the fixed DNA, removing unpaired RNA and unspecifically hybridized RNA, by treatment with RNase. The membrane filter manufactured by Schleicher & Schuell (Type B) is found to be the most suitable one. The most convenient method for the preparation of single stranded DNA is to treat it with alkali at one-tenth molarity (pH∼13) followed by neutralization after 10 min. The loading is done by filtering the denatured DNA solution (in 2×SSC) through nitrocellulose membrane. The filtration should be done at a slow speed to ensure efficient loading. DNA can be irreversibly retained in the filter by drying the filters at moderate temperature. It is better to dry the filters overnight at room temperature followed by heating for 2 hr at 80°C. under vacuum. Successful irreversible fixation of DNA is better achieved by filtration than by spotting. The size of DNA has no effect on its retention on the filter, though larger pieces are somewhat readily fixed. Loading of DNA is better in 6×SSC than in 2×SSC. Once DNA is

fixed, it does not get loose during hybridization or subsequent washing. DNA immobilized on the filter is fully available for hybridization. The extent of hybridization is the same with DNA in solution and that in immobilized form. Hybrids are formed by immersing the DNA filters in 5 ml. of 2×SSC or 6×SSC containing ^{32}P-RNA in vials with screw cap and incubating at the desired temperature (generally 66°C.) without shaking. After incubation, the vials are chilled in ice bath. To eliminate RNA 'noise', the filters are removed from the hybridization fluid, washed on both the sides with at least 50 ml. of 2×SSC and then incubated for 1 hr in 2×SSC with RNase (20 γ/ml.). After RNase treatment, the filters are again chilled and rewashed on both the sides as before. Washings are done by filtration under suction. The filters are finally dried and counted. The volume of the hybridization fluid can be minimized to 0·5 ml. by using small conical tubes and rolling up the filters. The DNA-RNA hybrid becomes unstable on prolonged incubation. The unstability of the hybrid is more when the hybrid is formed in the liquid than on membrane filters.

The modifications of the technique discussed above are mostly aimed at making the detection of hybrid formation sensitive and reliable. None of them is suitable for isolation purposes. The density gradient centrifugation can be successfully employed for this purpose, but involves long periods of centrifugation. Therefore, Hayashi *et al.*[44] employed the method of chromatographic detection and isolation of DNA-RNA hybrids on an MAK column. The method has the advantages of wide range in capacity and the high resolution achieved.

Applications of the Hybridization Technique

The DNA-RNA hybridization technique has been extensively employed for solving a large number of problems in molecular biology. Specific hybrid formation between DNA and RNA has been extensively used as a test for complementarity of base sequences between these two macromolecules. This demonstrated the involvement of DNA as the template for the synthesis of various types of RNA. As mentioned earlier, the DNA-RNA hybridization technique was first successfully used by Hall and Spiegelman[16] to demonstrate that the RNA synthesized in *Esch. coli* after T2 infection has base sequence complementary to that of phage DNA and not host DNA. The synthesis of the DNA-like RNA is not a speciality of the phage-infected host. It is also present in uninfected bacteria[5,26].

Due to complementarity of base composition between DNA and mRNA, the involvement of DNA in the synthesis of mRNA was suspected before it could be demonstrated by the formation of specific hybrids between DNA and mRNA. The origin of rRNA linked to DNA was, however, not suspected in the beginning, as the base composition of rRNA was known to be at variance with the overall base composition of DNA. Yankofsky and Spiegelman[30] first established that the synthesis of rRNA, like that of mRNA, is dictated by DNA. They demonstrated the formation of specific hybrids between rRNA and DNA. The saturation level of hybrid formation between DNA and rRNA[31] indicated that approximately 0·3 per cent of the total genome of *Esch. coli* has the information for rRNA. Non-ribosomal RNA, even from the same species, does not compete with rRNA during hybridization with DNA. The two types of rRNA (16S and 23S) have similar base composition. Therefore, it was natural to assume that they would have the same origin. But the absence of competition between 16S and 23S fractions of rRNA during the formation of hybrids[32] clearly indicated that the 16S and 23S fractions originate from two distinct regions on DNA. In the case of *Esch. coli*, 0·186 per cent of the total DNA hybridizes with 23S RNA and 0·117 per cent with 16S. In the case of *B. subtilis*, however, the values obtained for the two types of rRNA (23S and 16S) were found to be different (0·25 and 0·13 respectively)[45]. In the case of HeLa cells, considerable competition (about 60 per cent) has been reported[46] between two types of rRNA (28S and 18S). Such competition may also result due to contamination of the 18S fraction with the degradation products of the 28S fraction.

Taking advantage of the synchronous replication of chromosomes during the germination of the spores, the position of the rRNA gene on DNA was mapped by Oishi and Sueoka[45]. The hybridization between DNA and rRNA was used as a test for the transformation of rRNA marker. The other markers used were nutritional markers, such as adenine, threonine, leucine and methionine. The transfer of these nutritional markers was followed by the usual transformation technique. By following the sequence of transfer of different markers they could map the gene for 16S and 23S RNAs between two groups of adenine markers and proximal to the threonine marker.

It is also possible to ascertain whether there is only one or more than one set of informations in DNA for these RNAs, if the equivalent molecular weight of DNA, the saturation values of that species of RNA and the molecular weight of RNA as well are known. There is evidence to show that RNA is formed complementary to one of the two DNA strands in any given region. Therefore, the equivalent molecular weight of DNA should be divided by 2. The number of gene copies for ribosomal RNA is 5-20 in the case of bacteria, about 100 in the case of drosophilla and chicken and 1000 in the case of HeLa cells. Though not much importance need be attached to the rigidity of the number of gene copies, yet it does indicate the presence of multiple gene copies.

All the available data indicate that 0·3 per cent of the genome is involved for coding rRNA in the case of bacteria. There are multiple sites for both the types of rRNA and the densities of DNA-RNA hybrids suggest that they are clustered rather than scattered throughout the genome. In the mammalian system, a number of observations implied that nucleolus is the site of protein synthesis. There is no rRNA synthesis in a lethal anucleated mutant of *Xenopus laevis*[47]. Attempts were made to compare

the hybridization of nucleolar and non-nucleolar DNAs with rRNA[48,49]. But as it is rather difficult to prepare nucleolar DNA free from any non-nucleolar DNA, the results obtained were not decisive. To overcome these difficulties, different mutants of *Drosophilla melanogaster* possessing different doses of nucleolar organizer were produced. Hybridization was attempted between DNA derived from these stocks and isotopically labelled rRNA. The amount of RNA hybridizable per unit of DNA was found to be proportional to the number of nucleolar organizers (NO) per genome[50]. The proportionality between the amount of RNA hybridized and the number of NO regions confirmed the localization of rRNA genome in the NO region.

The formation of specific hybrids has been used to study the relationship between DNA and rRNA in plants as well[51]. The amount of DNA containing the code for rRNA varies over a 10-fold range among different plant species. Plants which show remarkably high hybridization between DNA and rRNA, indicated the presence of extra non-chromosomal DNA (satellite DNA). The satellite DNA isolated from pumpkin has been found to be greatly enriched with rRNA coding material.

As mentioned earlier, Goodman and Rich[35] as well as Giacomoni and Spiegelman[34] almost simultaneously demonstrated the formation of specific *Esch. coli* DNA-sRNA hybrids and concluded that DNA is also the primary site for the manufacture of sRNA. The saturation values obtained independently by these two groups of workers were very similar (about 0·02 per cent). Assuming that each amino acid is coded by only one triplet and decoded by only one sRNA, there would be 20 different sRNA molecules. Then the plateau obtained would have been 0·01 per cent, considering equivalent molecular weight of the *Esch. coli* genome as 4×10^9 and the average molecular weight of sRNA as $2{\cdot}5\times10^4$. The fact that the plateau obtained for sRNA hybridized with DNA is 0·02, i.e. twice the expected value, was in favour of degeneracy of the code predicted from the genetic experiments of Crick *et al.*[52]. Using almost the same techniques as is employed for determining the location of rRNA gene, Oishi *et al.*[53] demonstrated that the gene for sRNA is located between the two adenine markers as in the case of rRNA gene. The sRNA loci are distinct from those of rRNA, though they are very close to each other.

For a long time it was not understood whether both the strands or one of the two strands of DNA carried the genetic message. The DNA-RNA hybridization technique first provided the correct answer[54]. Analysis of mRNA synthesized under *in vitro* conditions as catalysed by DNA-dependent RNA polymerase indicated that RNAs complementary to both the strands are formed. On the other hand, the results of analyses of mRNA produced *in vivo* favoured the transcription of only one of the two strands. Though bacteriophage $\phi x174$ DNA is single stranded, double stranded DNA is at first formed following phage infection. This double stranded structure is referred to as the replicating form (RF). RF can be isolated in circular form and disrupted into linear structure, if necessary. RNA was synthesized under *in vitro* conditions using either circular or linear disrupted RF as the template. Both the types of RNA were used in hybridization experiments with RF DNA (double stranded) and mature phage DNA (single stranded). RF hybridizes readily with RNA synthesized either on circular DNA or linear DNA template, whereas mature phage DNA hybridizes with RNA synthesized on linear DNA only. RNA synthesized on circular DNA as the template did not hybridize with mature phage DNA. These experiments provided two important clues. The mature phage DNA does contain the genetic message. The complementary strand present in RF, which is produced after phage infection, is the one which contains the genetic information. Further, in the case of circular form only one of the two strands is transcribed, whereas in the case of linear disrupted structure both the strands are transcribed. Apparently, the circularity of DNA controls the transcription of the message from that strand only which contains the true genetic information. These evidences favour the storage of message in only one of the two strands of DNA.

The situation was found to be somewhat different in the case of λ DNA. The nearest neighbour base analysis of the RNA produced by RNA polymerase using λ DNA as the template showed unequal frequencies of complementary bases, suggesting that both the strands are not being copied at the same sites[55]. This agreed with the genetic evidences obtained by Hogness *et al.*[56] and Eisen *et al.*[57]. More confirmative evidences were obtained by hybridization of mRNA (synthesized either *in vitro* or *in vivo*) with physically separated intact complementary strands of λ DNA[58]. The ability of λ mRNA to hybridize with both the strands of DNA indicates that both the strands are involved in message production. Similar experiments were carried out by Szybalski and his coworkers[59] who arrived at the same conclusions.

Another area in which the DNA-RNA hybridization technique has been successfully utilized is the expression of early and late functions of the phage genome following entry into the host cells. Some viral specific enzymes are synthesized within short period after infection, whereas some enzymes are produced at later stages. The mode of regulation of early and late proteins following infection of *Esch. coli* by T-even phages has been studied by Hall *et al.*[60]. If the regulation occurs at the transcription stage, mRNA synthesis and protein synthesis should run parallel, otherwise there would be synthesis of late mRNA in the early periods too. Hall *et al.*[60] studied the competition between early and late RNAs synthesized after T2 infection, for complexing with DNA. Their results indicated that the genes concerned with the early function are transcribed at both the early and the late stages, while the late function genes are transcribed at the late stage only. With the help of T4 mutants deleted in the rII region (i.e. early function) or endolysin (i.e. late function) Bautz *et al.*[61] proposed, on the basis of hybridization experiments, that all the genes of the phage are transcribed once after entry into the host, and only those genes whose

messengers are translated into proteins continue to be transcribed. It has been observed by Friesen *et al.*[62] that almost all classes of early mRNAs are attached to polysomes late in infection and that the distribution of early and late mRNAs on late polysomes is similar.

The synthesis of specific mRNAs at various phases of the life cycle of the phage λ has been studied by Sly *et al.*[63] by the hybridization technique. λ-Specific mRNA was detected by the formation of RNase-resistant hybrid between ^{3}H-RNA and denatured λ DNA. A low level of λ-hybridizable RNA is found in non-lysogenic uninfected *Esch. coli*. In lysogenic strains of *Esch. coli* carrying the prophage λ, the fraction of ^{3}H-RNA that hybridizes with λ DNA is double of that found in non-lysogenic cells. These results suggested that at least some prophage genes function to direct mRNA synthesis. Similar results have been obtained by Attardi *et al.*[64]. After induction of the prophage by mitomycin C there is barely detectable increase in DNA level up to 30 min. However, after 50 min., the mRNA level is dramatically increased. On infecting non-lysogenic cells with virulent strains of λ(λV), 30- to 40-fold increase in mRNA synthesis can be observed within 2 min. of infection. This elevated level persists for nearly 20 min. and then it rises still further (another 4-fold increase).

Partial separation of DNA corresponding to rII marker of phage T4 has been achieved by Mazaitis and Bautz[65] by fractionating RNA from different rII mutants through double layer nitrocellulose column (in which DNA has been trapped) and subsequently using that RNA for hybridization with DNA.

DNA-DNA Hybridization

It is natural to expect that one class of DNA should hybridize with another class in case there is base complementarity. The DNA-DNA hybridization is, however, beset with many difficulties and the technique has not been developed fully.

DNA-DNA hybrid formation was used to study complementarity between host and phage DNAs by Cowie and Hershey[66] in connection with the establishment of lysogeny of phage λ with *Esch. coli*. The method adopted was more or less the same as the one used by Bolton and McCarthy[40] for the formation of DNA-RNA hybrids using DNA agar columns. Using this technique, some light has been thrown on the extent of specific sites on λ DNA for interaction with the host DNA[64]. λ DNA molecules were reduced to about 150 single stranded fragments which were tested for hybridization with *Esch. coli* DNA trapped in agar gel. Reactive fragments, about 50 or so, are derived more or less equally from the left, right and central portions of the molecular length, indicating that there are at least three or probably more sites on the λ DNA molecule that can interact with *Esch. coli* DNA.

To ensure rapid and sensitive assay, attempts have recently been made for detecting DNA-DNA hybrids on membrane filter by Denhardt[67] and Warnaar and Cohen[68]. They tried to develop the technique by modifying Gillespie and Spiegelman's[43] method of DNA-RNA hybrid detection on membrane filters. Denhardt[67] found that by preincubating the filters in an albumin solution, nonspecific sticking of denatured or single stranded DNA can be prevented, but the specific annealing of denatured DNA to complementary DNA previously bound to the filter is not blocked. This technique has been used to examine the kinetics of synthesis of single stranded ϕxDNA in ϕx-infected cells. To develop the assay of DNA-DNA hybrids on membrane filters, Warnaar and Cohen[68] utilized the observation of Klamerth[69] that single stranded DNA can be eluted from nitrocellulose with buffers of low ionic strength and high *p*H, whereas DNA hybridized cannot be eluted. Though the technique is still in a stage of development and needs further improvement, it has the advantage of avoiding the intermediate step of making RNA complementary to DNA. A combination of the methods of Denhardt[67] and Warnaar and Cohen[68] will probably yield better results.

Hybridization between the nuclear and kinetoplast DNAs of *Leishmania erriettii* and nuclear and mitochondrial DNAs of mouse liver has been studied by du Buy and Riley[70] employing the DNA-agar technique[40] and the membrane filter technique proposed by Denhardt[67]. The results obtained by either method indicate that the extranuclear DNAs contain nucleotide sequences, similar to nuclear DNA fragments, so that nuclear DNA fragments hybridize 40-70 per cent as effectively with extranuclear DNA as with the corresponding nuclear DNA. Hybridization between *Euglena gracilis* chloroplast DNA and nuclear DNA has also been attempted[71].

Summary

The development of the technique of DNA and RNA hybridization is reviewed and its advantages and disadvantages discussed. The application of the technique in solving a variety of problems in the field of molecular biology is described. The DNA-DNA hybridization is also briefly referred to.

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The Proteins of Liver Cell Sap

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THE pioneer investigations of many workers[1-9] during the period 1930-50 led to the fractionation of liver homogenates into the following main subcellular fractions by the application of differential centrifugation: (1) nuclei, sedimentable at the low speed of 600 *g* for 10 min.; (2) mitochondria, sedimentable at 10,000 *g* for 10 min.; (3) microsomes, requiring centrifugal speed of 105,000 *g* for 1 hr to sediment completely; and (4) cell sap, representing the unsedimentable constituents and also known as 105,000 *g* supernatant and soluble phase. The cell sap thus is the main stream for the metabolic commerce of the cell and is the milieu in which cellular formed elements persist and function.

The enzymes as well as the proteins which have no known enzymic functions (like structural proteins of mitochondria or histones of nuclei) in the three particulate fractions have been widely studied and reviewed. Even though recent studies on cell sap have shown that it has distinct enzymic composition and that it contains 40 per cent of the total proteins of the cell[10-12], little progress has been made on the characterization of the proteins contained therein.

Methods Used for the Resolution of Cell Sap Proteins

Electrophoresis on various supporting media like paper, agar, cellulose acetate, as well as in liquid media (moving boundary) has been used to fractionate liver proteins. Column chromatography has been found to give better resolution and to provide a preparative tool for enzymes.

Paper electrophoresis — Demling *et al.*[13-17] were perhaps the first to apply the electrophoresis technique to the examination of rat liver proteins. In earlier studies[13-15] they electrophorized the total extractable proteins from the whole rat liver, while later[16,17] they studied the proteins of nuclei, mitochondria and cytoplasmic supernatant separately. They obtained a resolution of proteins on these fractions into four major components, α, β and γ globulins and albumin. They also reported the quantitative distribution of these four protein fractions in the three subcellular fractions.

Adjutantis[18] reported the fractionation of microsomal free cell sap proteins into 5 fractions, using borate buffer of *p*H 8·6 and μ=0·18. He observed that freshness of the cell sap was important for electrophoretic resolution and that butanol treatment of the cell sap led to improved resolution into seven fractions. Gigante *et al.*[19,20] prepared extract of total soluble proteins of rat liver by freezing and thawing followed by centrifugation and dialysis and carried out electrophoretic study using Michaelis buffer. Pellegrino and Caravaglios[21] fractionated the centrifuged borate buffer extracts of rat liver into nine components and localized alkaline and acid phosphatases, esterase and β-glucuronidase in these fractions.

Kaplansky *et al.*[22] reported that by freezing and thawing rat liver homogenates in saline and centrifuging, they could increase the soluble protein content from 2 to 4 per cent. They could fractionate the protein extract thus obtained into 5 fractions (the electrophoretic mobility of which corresponded to those of serum protein fractions). Kaplansky *et al.*[23] also found that some of the liver protein fractions were serologically identical to serum albumin and others to β and γ globulin.

Agar gel electrophoresis — Goranov *et al.*[24] ground perfused liver with sand in 7 per cent NaCl and, after storing for 24 hr, centrifuged at 6000 r.p.m. for 2-3 hr and used the lipid layer-free supernatant for agar gel electrophoresis. Thirteen well-defined fractions were obtained after staining with Amido Black and were quantitated in a Zeiss extinction registrator. Kessell[25-27] prepared the extract in barbiturate buffer (*p*H 8·6) and carried out the electrophoresis. He found electrophoresis on agar to give better results than on filter paper. Other workers[28-34] carried out agar gel electrophoresis of total soluble proteins of rat liver, localized some enzymes in these separated fractions and studied their immunological characteristics.

Cellulose acetate electrophoresis — Mullan *et al.*[35] removed most of the enzymatic proteins from rat liver supernatant by precipitation at *p*H 5·1-5·2 and examined the remaining proteins by cellulose acetate electrophoresis. They obtained resolution into five fractions by unorthodox anodic application as against four with cathodic application. They suggested the site of application as an important factor for the resolution of proteins.

Moving boundary electrophoresis — Sorof *et al.*[36,37] were among the first to study the soluble proteins of rat liver by Tiselius electrophoresis. Since then, there have been other studies[38-41] on liver soluble proteins by moving boundary electrophoresis. Anderson *et al.*[42] studied the distribution of catalase, β-glucuronidase, acid phosphatase, esterase, adenosine deaminase, nucleotide phosphorylase and glutathione reductase in various protein fractions of rat liver supernatant (105,000 *g*) obtained on electrophoretic resolution.

Column chromatography — Moore and Lee[43] carried out the chromatographic separation of rat liver supernatant (105,000 *g*) proteins on DEAE-cellulose and localized many of the enzymes in the separated fractions. Their study pointed towards the possible existence of more than one form of an enzyme in the supernatant fraction, since they obtained the same enzyme activity in two vastly separated fractions.

Extensive chromatographic studies of soluble proteins of liver on ion-exchange column have been carried out as part of enzyme purification

procedures. However, initial extracts are often made by methods which do not minimize damage to subcellular components. In addition, interest in isolation leads to the development of isolation methods giving maximum purification of the enzyme in question and not to techniques for achieving maximum resolution of the entire mixture of proteins. The latter problem has been studied in detail by Bond[44] and an elution programme for high resolution separation of rat liver cell sap proteins on DEAE-cellulose column developed. This work led to the discovery of a protein present in the liver of female rat but either completely absent or present in small amounts in male liver[45-47].

Cell sap proteins in abnormal states — There are characteristic changes in liver soluble protein electropherograms, when rats are exposed to nutritional or other experimental stresses. Electrophoretic studies have been conducted on alterations in liver soluble proteins in thiamine deficiency[48], regenerating liver[49-51], traumatic shock[52], in patients with malignant blood diseases[53], radiation sickness[54], ischaemia[55], protein deficiency, $CHCl_3$ and CCl_4 poisoning[56-59], starvation[60], vacuolar degeneration and cloudy swelling[61,62], and after feeding carcinogenic dyes[63,64].

These studies, though carried out mostly on total extractable proteins of liver and not on cell sap, gave many important results. It was established that certain specific protein fractions get altered in a particular kind of damage; study of these specific proteins may be important in elucidating the metabolic derangements brought about by specific stress conditions.

Almost all the reports dealing with the fractionation of soluble proteins of liver represent the total extractable proteins of liver rather than the proteins of cell sap. These studies have, therefore, included some proteins of particulate fractions also. Furthermore, with the introduction of new techniques of protein fractionation like acrylamide gel electrophoresis (which has much higher resolving power, is free from denaturation and is least time-consuming), these reports need reinvestigation. Better resolution is more likely to help in the identification of multiple forms of enzymes and in the separation of conjugated proteins. Besides, with these better techniques, studies on the relative alterations of various protein fractions of supernatant in various pathological states will give more precise information.

Thus, using the acrylamide gel electrophoresis it has been possible in this laboratory (unpublished results) to fractionate the supernatant proteins into 16 well-defined fractions, the highest resolution obtained so far on any medium. Xanthine dehydrogenase was localized in one fraction, while aspartate aminotransferase was present in two vastly separated ones, suggesting the occurrence of multiple forms. This technique, no doubt, will be of considerable help in looking for isoenzymes and multiple forms of various enzymes present in the 105,000 *g* supernatant of liver and other organs. It was also observed that some of these fractions like xanthine dehydrogenase disappear or get preferentially decreased in protein depletion, while others remain unchanged. Thus, it is possible to characterize the proteins which are not altered during protein deficiency and are perhaps more necessary for metabolic functions as contrasted with other proteins which disappear completely and which, therefore, apparently can be dispensed with relatively more easily (unpublished observations).

Enzymic composition of cell sap — Several excellent articles reviewing the results of studies on the distribution of various enzymes in different subcellular fractions isolated by differential centrifugation have appeared[65-68].

Among the important enzymes predominantly present in the cell sap are those associated with glycolysis[69-78], hexose monophosphate shunt[79,80], amino acid synthesis[81], glutathione metabolism[82,83], amino acid activation[84], and thymidine incorporation into DNA[85]. Many enzymes concerned in the breakdown of purines and pyrimidines[86-94], transformation and synthesis of porphyrins[95], biosynthesis of cholesterol[96-99], phosphorylation of thiamine[100,101], vitamin B_6[102-104], glycogen metabolism[102-104], etc., are almost completely localized in this fraction.

The supernatant fraction contains some enzymes which are also present in particulate fractions. In a few cases, the bimodal distribution pattern has been attributed to release from particulate fractions during homogenization, adsorption of soluble enzymes or to other artifacts. In other cases, it has been convincingly shown that the two enzymes are distinct and separate proteins. Thus, aspartate aminotransferase[105-110], alanine aminotransferase[111,112], malate dehydrogenase[113-117], alkaline phosphatase[118-122], phosphoprotein phosphatase[123], acetyl CoA hydrolase[124], aconitase[125] and aldolase[126,127] belong to the latter category, while NAD and FAD pyrophosphorylases[128,129], fumarate hydratase[68,130-132] and catalase[133] belong to the former class.

Still, however, there are many enzymes[133-138] having bimodal distribution patterns for which such studies have not been made. These will be important in correlating the subcellular sites or organs damaged in various pathological states with increased release in serum as has been done for aspartate aminotransferase in this laboratory[139].

In spite of these studies, it is not yet known as to what percentage of soluble proteins is accounted for by known enzymes. Unless such a balance sheet is drawn, it is difficult to know whether there are proteins in this fraction which have functions other than enzymes, as in plasma. However, recent studies have pointed towards the existence of some conjugated proteins which might play a role in the transport and storage of vitamins and lipids and of some basic proteins which might act as regulators in the liver cell sap.

Occurrence of Conjugated Proteins in Cell Sap

The supernatant fraction contains a high percentage of many of the vitamins[140-149]. The binding of some of the vitamins with proteins has been shown[148], but it is not known whether they bind

specifically to a single protein. If so, a protein-vitamin complex has not been purified and it is also not known whether the combination is enzymatic and what factors regulate it.

Studies on lipid composition of supernatant[150-158] have shown that it contains more than 50 per cent total neutral fat and 30 per cent total cholesterol. Yet, there is one report[159] suggesting the occurrence of lipoprotein in cell sap, since the lipids of this fraction were incompletely extracted in ether and were precipitated along with proteins by trichloroacetic acid. It is obvious that the lipoproteins of this fraction need further study. Similarly, there are no reports on the occurrence of glycoproteins in this fraction.

Thus, in this laboratory, using paper electrophoresis, the lipoproteins of this fraction have been resolved into 5 distinct fractions, their chemical analysis done and their metabolic significance in relation to fat transport demonstrated[160]. The possible occurrence of glycoproteins and vitamin-protein complexes is being currently investigated.

Basic Proteins of Cell Sap as Possible Regulators

The role of basic proteins of cell sap as possible regulators has been suggested, since (i) they combine specifically with carcinogens[161-164]; (ii) they inhibit cell multiplication cytostatically[165]; and (iii) some of the proteins of cell sap can permeate into nuclei[166,167]. However, the resolution, purification and isolation of the individual basic proteins of the cell sap have not been done. Thus, when the paper electropherograms (*p*H 8·6) of rat liver cell sap proteins were examined under ultra-voilet light, a red fluorescent band which had moved towards the cathode was observed. This fraction was undialysable and could be stained with protein stains such as bromophenol blue. This basic protein was purified from rat liver cell sap by treatment with calcium phosphate gel, and the supernatant obtained passed through DEAE-cellulose equilibrated with borate buffer of *p*H 8·6. In the final step, the eluate was dialysed, freeze-dried and chromatographed on CM-cellulose equilibrated to *p*H 6·2 with phosphate buffer. The spectral characteristics of this protein were studied and depending on them, a method of assay was developed. This protein could inhibit *in vitro* multiplication of mouse fibroblast cultures at a concentration of 400 μg./ml. medium. The nature of the fluorophor which is different from porphyrins, the only other red fluorescent compound known to occur in nature, as well as the possible role of this basic protein as a regulator are being currently examined (unpublished observations).

Immunochemical Analysis of Cell Sap Proteins

Immunochemical and immunoelectrophoretic analyses of liver cell sap proteins have been reported by many workers[168-174]. The results support the view that in liver, a relatively small number of proteins are present in rather high concentration against a background of many, which account for the biochemical versatility of the organ. A minimum of five immunologically distinct, non-plasma proteins can always be observed in rat liver cell sap. The h_2 class of proteins described by Sorof *et al.*[163] is one of these. Using immunoelectrophoresis, 7-10 lines are observed with the rat liver soluble phase. During regeneration three lines were frequently absent, one was always absent, three showed no change and the intensity of two increased.

The correlation of immunologically identified proteins with fractions isolated by centrifugation, electrophoresis, chromatography and with enzyme activity known to occur in the cell is urgently needed.

Effect of Heat on Cell Sap Proteins

Proteins of rat liver cell sap are surprisingly unstable to heat. At 40°, 10 per cent of the total protein nitrogen is precipitated in 2 hr[174]. The observation that a sizeable percentage of the soluble proteins of rat liver precipitate *in vitro* during incubation at temperatures not lethal for an intact animal raises several interesting questions. Though the physiological significance of this finding is not yet known, the finding that several biochemicals inhibit precipitate formation is of interest[175].

When the clear ultracentrifuged extracts of rat liver are allowed to stand in cold, a variety of structures, many of which resemble components of liver cells, are formed[175]. According to Anderson *et al.*[175], these structures, which resemble cell components, exist in equilibrium with their constituent molecules in solution and that mechanism similar in part to those involved in structure formation in solution may also be there in the living cell.

The studies hitherto on cell sap proteins have indicated the occurrence of (i) basic proteins which might have regulator roles, (ii) lipoproteins, glycoproteins and vitamin-protein complexes and (iii) proteins which may have functions other than as enzymes. With the better techniques now available further resolution and characterization of the proteins of the cell soluble fraction may be expected.

Summary

Recent methods for the resolution and characterization of proteins contained in the 105,000 *g* cell sap have been reviewed and their application for studying (i) the localization and purification of enzymes and isoenzymes, (ii) relative changes in the protein fractions resulting from various abnormal states, and (iii) binding of vitamins, cofactors, lipids and carbohydrates are discussed.

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REVIEWS

NONLINEAR ELECTRON-WAVE INTERACTION PHENOMENA by Joseph E. Rowe (Academic Press Inc., New York), 1965. Pp. xiv+591

This is essentially a research monograph presenting details of theoretical analyses of the interaction between well-defined beams of charged particles (electrons and/or ions or plasmas) and propagating electromagnetic waves in electron beam devices. The analyses aim at determining the upper limits, set by the nonlinearities in the electron-wave interaction process, on the performance of a complete range of electron beam devices. Chapter II outlines the basic approach to the handling of one of the most troublesome manifestations of the nonlinear behaviour, namely the overtaking which takes place in the electron stream. It consists in considering the electron beam, not as a charged fluid (which would have a multivalued velocity function if overtaking takes place) but as a collection of charge bunches whose motion is described by the Lorentz force equation. The next chapter is concerned with setting up equivalent circuits for propagating electromagnetic waves in various types of devices, the circuit equations and parameters being obtained from Maxwell's equations and boundary conditions appropriate to each device. The general theory is concluded with the derivation of space charge forces for various beam geometries in Chapter IV. Chapters V to XI present analyses, in great detail, of specific types of devices (klystrons, travelling wave amplifiers and backward wave oscillators — with only longitudinal static magnetic fields or with crossed electric and magnetic fields — and travelling wave energy converters). The book concludes with an evaluation of the efficacy of various special schemes (phase focusing, prebunching of the electron beam, etc.) in improving the operating characteristics.

The material presented in the book is essentially a systematic compilation of recent work in this field, a significant part of which is due to the author himself. The point of view is that of a practising design engineer, who wants directly usable numerical results from the theoretical analysis; a substantial part of the space in the book is taken up by graphs depicting the dependence of the characteristics of the devices on various parameters, the graphs themselves being electronic computer solutions of the complicated equations. While the treatment presupposes good familiarity with the devices and their small signal theories, the exposition is systematic, detailed and lucid.

This volume will serve as a valuable reference work for specialists in the microwave engineering field, particularly those concerned with design aspects, and perhaps as a text-book for an advanced graduate level course.

P. M. MATHEWS

HIGH SPEED PULSE TECHNOLOGY Vol. II — OPTICAL PULSES LASERS, MEASURING TECHNIQUES by Frank Früngel (Academic Press Inc., New York), 1965. Pp. xiii+477. Price $ 21.50 or 176*s*.

This is the second of the two volumes on high speed pulse technology. The first volume considered components and equipments for conversion of electrical impulse energy to heat, magnetic field, electric field, acoustic energy and electro-erosion for various practical applications. The second volume treats the topics of light flash production from capacitive energy storage, signal transmission and ranging systems by capacitive discharges and lasers, and impulse measuring techniques. Details are given of many components and equipments designed for high speed photography, lasers, signalling, cloud ceilometers, visibility meters, spectral sources and impulse monitors. The monograph contains a wealth of information gathered from scattered scientific literature of the past hundred years, author's own studies and contributions for the past twenty years and manufacturer's products presently available. The subject matter is of direct interest to scientists, engineers, and instrument manufacturers working in the fields of high voltages, spectroscopy, nuclear physics, aerodynamics, hydrodynamics, ballistics, explosives, microsurgery and manipulation, welding, meteorology, and signalling and ranging. The exposition is very clear with fundamentals, numerical design data, tabulations, figures, photographs and over one thousand references listed in the bibliography. Each topic covered is self-contained without having need to read the previous pages of the volume.

Principles of high speed photography are presented. For photography of rapid movements the exposure is given by mechanical methods, electronic shutters and flash sources. With flash sources it is possible to have exposures in the range of millisecond to nanosecond durations with relatively simple equipment. The principal methods of photography described are the direct shadow by point source, the Schlieren method which shows large contrast in light for slight changes in refractive index in air-flow patterns and the tracer method in which ionization trails of successive sparks show supersonic air-flow patterns. Many other tracer and synchronizing methods for photography and cinematography of rapid movements are described. Some set-ups described are simple and inexpensive for illustrating the principles to students. The design data for spark and flash tubes are given exhaustively from the following points of view: intensity of light output, duration of light, duration of after-glow, stability of light output, spectra content of light, energy conversion efficiency, incorporation in optical system as point line or distributed source, and high frequency operation. The pulsed ruby laser and the Q-switched ruby laser designs and their numerous applications are

given. The applications discussed at length are for welding, signalling and ranging.

In the chapter on 'Signal transmission and ranging' we have design data of atmospheric attenuation daylight interference, signal to noise ratios obtainable with different photodetectors and filters. The practical equipments using spark source discussed are for signalling to 30 km. distances in line of sight, signalling for bypassing in traffic, cloud ceilometers, visibility meters for air traffic, and secret military movements using invisible ultraviolet portable signal equipment.

In the chapter on 'Impulse measuring techniques', we have extensive data, tables and graphs to utilize spark gaps as standards for measurements, taking into effect air density, humidity, duration of impulse, polarity, ageing, etc. Other measurement techniques given are oscilloscopy, Hall probes for magnetic impulses, photo multipliers, and image converters for light impulses, infrared monitoring, X-ray monitoring, luminescence and shock waves.

To increase its effectiveness as a reference volume, it is to be hoped that future printings and editions will expand the subject index to more topics. There are a few minor printing errors which can be easily corrected by the reader from their context.

R. S. V. SITARAM

DIMENSIONAL ANALYSIS AND HYDRAULIC MODEL TESTING by H. M. Raghunath (Asia Publishing House, Bombay), 1967. Pp. v+112. Price Rs 15.00

The book is a laudable attempt on the part of an Indian publisher to bring out an authoritative monograph on a branch of engineering research on which several hydraulic and other research institutes in the country are actively engaged.

The author, after a very brief introduction, acquaints the reader with dimensions and dimensional analysis. Under dimensions and units, the significance of the 'physical dimensions' as distinct from what it means in common usage and the 'law of derived dimensions' should have been elaborated in more detail. Although Table I gives a comprehensive list of the unit systems, short explanatory notes on the mass-based and force-based systems and particularly a general treatment of the conversion factor would have been useful. The subject of dimensional analysis itself has been very summarily treated. Buckingham's π theorem is stated without proof; at least where to look for the proof could have been indicated. The advantages of the dimensional analysis are listed at the end of the chapter; some illustrations of these uses would have been enlightening. Chapter 4 on hydraulic similitude is quite comprehensive. However, a short paragraph on the scale effects, particularly in models of hydraulic structures, should have been added as it is well known that such an effect exists mainly due to variation in the growth of the boundary layer with size.

In the next chapter on non-dimensional factors, a paragraph explaining the cavitation phenomena and an illustrative example on the technique of non-dimensional plotting could have been added. Otherwise it is a very well written chapter. Chapter 6 deals with river models. The various river modelling techniques have been explained in detail, but a more comprehensive treatment of the principles of model verification should have been there as the time scale of movable bed river model is invariably developed in the process of the model verification. The recent techniques of movable bed modelling due to Einstein and Chein, Bogardi, Komura and others have not been adequately mentioned. On page 63, last line, the recommended figures of US Waterways Experiment Station on Reynolds' numbers differ widely from the recommendations from the same source as quoted by Allen in his book *Hydraulic model.*

As regards tidal models, the distortion of 8-10 is now realized as too high; for reliable results the distortion should not exceed 5. In Eq. 12(c) in Chapter 7, the sign of inequality should be $\leqslant$ in place of $<$.

The treatment of hydraulic machinery models is rather sketchy. Only the relations by which the various values for the actual sized turbines are calculated from the principles of similarity have been explained.

Few more photographs and diagrammatic sketches might have been profitably added to the chapter on 'Techniques and operation of hydraulic models'. The last chapter on model prototype conformance is too short to be useful.

There are some serious printing mistakes; πs have been put as π-S and V and v have been used indiscriminately to signify velocity. Arithmetic of some of the illustrative examples has been checked by the reviewer and at least one is incorrect.

In spite of the above remarks, I should acknowledge that the book is an excellent practical introduction to the practice of hydraulic model technique. The remarks are really suggestions for improvement in the subsequent editions.

S. K. NAG

SPECTROSCOPIC TRICKS by Leopold May (Plenum Publishing Co. Inc., New York), 1967. Pp. xiv +333. Price $ 9.50

Spectroscopists and analysts would particularly welcome the present compilation which gives in one volume the tricks and notes that appeared in *Applied spectroscopy* since 1959. Several of the techniques have been already in use in different laboratories either in the same form or slightly modified. The notes have been arranged in seven well-defined areas of spectroscopic work, while the last section is of a general nature and caters to many areas of spectroscopic work. The reviewer finds several useful contributions on emission and infrared spectroscopy that are distributed under sections I and II. Raman spectra are not as well covered as they ought to be. There are number of innovations which would be novel and improve the efficiency of a particular process. The working spectroscopist will be very happy to have all these notes in such a volume quite handy. The compilation is indeed a worthwhile effort.

N. A. NARASIMHAM

A SYMPOSIUM ON CHEMICAL ADDITIVES IN FOOD, edited by R. W. L. Goodwin (J. & A. Churchill Ltd, London), 1967. Pp. viii+128. Price 20*s*.

For the whole world and particularly to the developing countries with their increasing population, the maximal utilization of the available food is of utmost importance. Food technologists solve these problems by processing and improving methods of packaging, storage and transportation of raw and processed foods. Besides physical and biological processing, addition of chemicals to improve the appearance, flavour, texture and storage properties of foods has been a significant development. Prime consideration in all these developments has been safety and quality of the foods. And yet, one often hears of the horrors of addition of 'chemicals' in food — the devitalizing effect of processed foods, the insipid foods raised with chemical fertilizers. J. G. Davis' excellent introductory paper to this symposium on Chemical Additives in Food, held at Coventry Technical College in 1965, gives a very rational and convincing answer to the fallacious arguments of natural food fadists.

This is followed by six other papers on different aspects by expert authors from industry and research institutions. The paper on 'Food preservatives' discusses chemical preservatives, including antibiotics, in actual use and new potential ones. 'Biological evaluation of food additives' shows that an extensive and thorough study is made today before any additive is considered suitable in the food industry. The following paper on 'Antioxidants' neatly summarizes the current understanding of the mechanism of autoxidation of fats and their prevention, while that on 'Food packaging — the unintentional additive' shows clearly how conscious the packaging materials industry is of the toxic and flavour problems from some of the minute quantities of chemicals used by them. The last two papers on 'Food flavours' and 'Food colours' are very interesting from the consumer standpoint of making the food more appetizing. The analytical and synthetic developments in the field of flavours have been very impressive, showing the extreme complexity — hundreds of compounds in one flavour — of natural flavours and the impracticability of listing and controlling flavour compounds. The synthetic colours create a real problem in the food industry in spite of permitted lists, legislation being in force in many countries. There is need for international cooperation for evaluation and producing an international permitted list of colours and their standards. It is encouraging to note the formation of the Codex Alimentarius Commission on Food Standards, sponsored jointly by the Food and Agriculture Organization and the World Health Organization, which will assure safety and improved quality from the use of chemicals in food.

The book being reasonably priced and giving excellent critical reviews of the different aspects by authorities in their respective fields will be welcomed by all concerned with food technology — students, research people, processors and manufacturers of food additives.

V. S. GOVINDARAJAN

THE STRUCTURE AND STRENGTH OF METALS by A. R. Bailey (Metallurgical Services Laboratories Ltd, Betchworth, UK), 1967. Pp. viii+122. Price 32*s*.

The Metallurgical Services Laboratories Ltd, Betchworth, UK, market metallographic specimens prepared under controlled conditions. These specimens are accompanied by a book which gives full practical instructions and continuous commentaries with typical results on each specimen sequence. The object of the present book is to outline the way in which the strength of metals (at atmospheric temperature) varies with their microstructure. Five groups of specimens specially prepared are provided. The course is expected to provide a useful introductory material for those approaching the subject with an engineering bias. For those with more of a physical or metallurgical interest, the course offers an easy starting point for work on microstructure without involving any great knowledge of transformations in metals. For those interested in material science, the course provides a valuable exercise on the relations between properties and the structural morphology of crystalline aggregates applied to materials that have been rigorously studied and are easy to handle and prepare. The printing of the book has been of a very high standard and the microstructures are very beautifully illustrated.

A time has now come for us in India to fabricate our own laboratory equipment and other laboratory requisites as illustrated in this book so that a lot of foreign exchange may be saved. Under a well-coordinated policy, books such as this one together with the specimens could be prepared in various branches of engineering by laboratories or institutes of good standing. Such efforts will go a long way in making us self-sufficient in a number of laboratory requirements and we would have to look every now and then to the government to spare foreign exchange. We should, therefore, admire and imitate the type of service provided by the Metallurgical Services Laboratories Ltd.

G. S. TENDOLKAR

PUBLICATIONS RECEIVED

COMPARATIVE BIOCHEMISTRY OF THE FLAVONOIDS by J. B. Harborne (Academic Press Inc., London), 1967. Pp. viii+383. Price 90*s*.

CHEMISTRY AND INDUSTRY edited by D. G. Jones (Oxford University Press Ltd, London), 1967. Pp. ix+217. Price 20*s*.

UNIFIED CIRCUIT THEORY IN ELECTRONICS AND ENGINEERING ANALYSIS by J. W. Head & C. G. Mayo (Iliffe Books Ltd, London), 1965. Pp. 174. Price 42*s*.

1967 STEAM CHARTS (Edward Arnolds Publishers Ltd, London), 1967. Pp. vii+64. Price 60*s*.

SEMIMICRO QUALITATIVE ANALYSIS by F. J. Welcher & B. B. Hahn (East-West Press Ltd, New Delhi), 1967. Pp. x+497. Price Rs 10.00

THE CARCINOGENIC ACTION OF MINERAL OILS: A CHEMICAL AND BIOLOGICAL STUDY (Her Majesty's Stationery Office, London), 1967. Pp. xii+251. Price £ 2

NOTES & NEWS

A new nuclear method for determination of boron in glass

A new nuclear method of analysis has been developed to determine boron in glass. The method involves the measurement of the γ-ray produced on the capture of thermal neutrons by the element to be determined. The technique is comparable with non-destructive neutron activation analysis but has the advantage of determining certain elements which cannot be determined by the activation technique.

The masking of peaks in γ-ray spectra and the interference by other capture γ-ray activities are successfully avoided in the present method. The area under the photo-peak of a capture γ-ray spectrum of glass containing boron is directly proportional to the amount of boron present in the glass. While difficulties can arise in determining photo-peak areas when there are many such peaks in a complex spectrum, the area of the single photo-peak from boron in irradiated glass can be determined by simply summing the counts in the channels under it [*Glass Technol.*, **8** (1967), 154].

Fixation of radioactive wastes — A new method

A unique process envisaged for the final fixation of radioactive wastes from the fission processes [*Ind. Engng Chem., Proc. Des. & Develop.*, **7** (1968), 117] consists in reducing the volume of the liquid wastes, converting them into oxide or sulphate form and finally reducing the mass to an insoluble polysilicate by a thermite reaction. The thermite reaction, carried out as a batch operation, serves as a source of heat and also furnishes the silica to incorporate the radioactive substances into a polysilicate structure. Two preparation routes which differ in the chemical composition of the resultant thermite reaction are conceived.

In the oxide route the liquid waste is treated to provide a material which can be satisfactorily handled and the resultant feed evaporated to remove H_2O, HNO_3 and traces of nitrogen oxides. The concentrated waste is intermittently discharged to the thermite reaction vessel which is charged with a predetermined amount of thermite mix of Si and Fe_2O_3. If necessary silica can also be added as a moderator to provide the desired reaction rate and final product composition. The vessel is transferred into a hot cell and covered with a gas-tight cover through which the necessary gas, liquid and solid connections are made. The mixture of the concentrated waste and the thermite mix is heated to remove water. The dry salts in the reactor are covered with a fresh layer of thermite mix, which acts as a cap to trap the volatile ruthenium and cerium during denitration and ignition.

The furnace temperature is raised to 500-600°C. to decompose nitrates during which the volatile fission products are adsorbed in the cap. After denitration the thermite cap is ignited and the reaction proceeds downwards through the mass. The heat liberated during the reaction raises the temperature of the mass and the radioactive oxides present are fixed in the silicate matrix. Molten iron produced at this stage flows to the bottom of the reactor where it is solidified by cooling. After the completion of the reaction the reactor is removed from the furnace, sealed and transported to the disposal area. The off-gases from the evaporator and reactor are scrubbed countercurrently with dilute nitric acid and cooled to condense water and nitric acid. Non-condensed gas is passed through an adsorber-dryer in which any remaining radioactive gases are removed. Spent adsorbent from the dryer can be disposed off periodically by mixing with the thermite charge to the reactor.

In the alternate sulphate route process, the salts present in the solution are converted to sulphates by the addition of sufficient H_2SO_4 prior to fixation. In this the thermite mix consists of Si and SiO_2, the latter being added to prevent greater temperature rise. Oxygen is supplied by the metal sulphates, chiefly $Al_2(SO_4)_3$, from the prepared waste. Since little or no nitrate is present in the charge, cesium and ruthenium do not volatilize during either drying or ignition.

The reaction mixture is ignited and the heat of reaction, which is 2·5 times more than that in the oxide process, leads to the fusion of the mass and incorporation of the radioactive materials in the silicate structure. Sulphur vapour produced is converted into SO_2 by air injection and SO_2 along with the off-gases is recirculated through the evaporator and recovery reactions.

The process is largely conceptual; the potential advantages like the high resistance of the final product to bleaching could be of importance.

Production of sorbitol using ammonia synthesis gas

Sorbitol, besides being an intermediate for the production of vitamin C, is an excellent moisture stabilizing agent and finds use as a substitute for glycerol and glycol in different industries. Presently it is produced by the catalytic reduction of *d*-glucose and also the high pressure, catalytic hydrogenation of *d*-fructose. A new high pressure hydrogenation process in which pure hydrogen is replaced by ammonia synthesis gas as the hydrogenation agent has been developed for the production of sorbitol [*Ind. Engng Chem., Proc. Des. & Develop.*, **7** (1968), 107]. In the process, which is to be used in conjunction with an ammonia plant, 2-4 per cent of Raney nickel is added to a 50 per cent dextrose solution. The mixture is blended with a 200 atm. pressure synthesis gas (75 : 25 hydrogen : nitrogen mixture by volume) drawn from the ammonia plant. The reaction mixture is passed through two in-line reactors at a space velocity of 1-3 kg./litre hr and with stepwise heating to 170°C. The hydrogen content of the synthesis gas is reduced only by 0·05 per cent; the gas after separation

from the liquid product and carbon dioxide is recirculated to the main ammonia plant. The catalyst is filtered out from the product-catalyst mixture and reused. The new process, besides eliminating the problem of hydrogen recirculation, gives a product of high and uniform quality with a high capacity. Hydrogenation by ammonia synthesis gas provides significant economic advantages by reducing the investment and production costs by about 24 and 80 per cent respectively.

New process for polyphosphate production

Polyphosphates are conventionally manufactured by the thermal dehydration and condensation of partially substituted orthophosphates. This process, besides being unsuitable for producing thermally unstable polyphosphates, involves loss of large amounts of energy. To overcome these disadvantages a new method based on the selective extraction of the desired fraction of a chain phosphate from superphosphoric acids by an organic amine has been envisaged. The superphosphoric acids consist of a mixture of phosphates of various chain lengths. The distribution of the various phosphate species in these acids depends on the P_2O_5/H_2O mole ratio. Tri-*n*-octylamine (TOA) which forms salt-like amine-phosphate complexes is chosen as the extracting agent. Under low acid concentrations TOA preferentially extracts the longer chain phosphoric acids. From the TOA-phosphate salt complex the required polyphosphates are obtained by the addition of the corresponding alkalis. Both the countercurrent and the fractionating extraction processes are envisaged [*Ind. Engng Chem., Proc. Des. & Develop.*, **6** (1967), 414].

Synthesis of macrocyclic compounds

A new, simple method for the synthesis of macrocyclic compounds from readily available cyclic ketones has been reported [*J. Am. chem. Soc.*, **90** (1968), 817]. By mild photolysis or thermolysis of appropriate ketone peroxides the desired macrocyclic hydrocarbons and hydrocyclic lactones (containing as many as 23 carbon atoms) are obtained in good yields. In addition, the reaction regenerates the ketone starting material. In the new synthesis, the dimeric- or trimeric-peroxide of any of the various cyclic ketones is obtained by reacting the ketone with hydrogen peroxide at room temperature. The peroxide precursors are then irradiated or heated to give directly a mixture of cyclic hydrocarbons and lactones. For photolysis a 4 per cent solution of the peroxide in methanol or benzene is irradiated with a 450 W. lamp for 3 hr. Thermolysis is effected by heating the peroxide in an evacuated, sealed ampule at 150°C. for 30 min. Alternatively, thermolysis can be carried out by running the peroxide through a continuous gas chromatograph in a variety of columns at 180°C. Though a particular reaction can be effected by both thermolysis and photolysis, the best yields are obtained in some cases by photolysis and in others by thermolysis. This procedure gives almost any macrocyclic compound provided the necessary peroxide precursor can be attained. The thermal or photochemical decomposition of the ketone peroxides is supposed to proceed through homolysis of an oxygen-oxygen bond analogous to that of alkyl peroxides and ozonides.

New simple method of micrurgy on living cells

A simple method has been developed, whereby micrurgy on living cells, such as nuclear transplantation and cytoplasmic injection in amoebae, can be performed with greater ease and rapidity than in the conventional methods. In the new method, the cells are placed on an agar surface and thereby the need for a micro-hook to hold cells and the use of mineral oil (liquid paraffin) to prevent the evaporation of the culture medium are dispensed with. The agar gel replaces the medium that evaporates and there is no damage to the cells during the period required for micrurgy.

Agar (0·6 per cent) in the cell culture medium is boiled, filtered and poured while hot on to clean slides or coverslips. As soon as the agar cools and gelates, cells can be placed on it and are ready for operations. Since a micro-hook is not used, it is necessary to place the cells in the desired positions beforehand. As a result, the cells cannot move and can, therefore, be easily distinguished by their relative positions during and after operation. The new method possesses several advantages: There is no hook to make and use, thus eliminating the most troublesome portion of the micrurgical work. As a result, the operation can be performed with much greater ease and rapidity than with other methods. For example, as many as 200 amoebae can be easily enucleated in 1 hr and 30 nuclear transplantations can be carried out during the same period under optimal conditions. The agar coated slides have also been found useful in immobilizing other motile cells, such as *Tetrahymena* and *Paramecium* for microscopic examination and manipulation of these cells [*Nature, Lond.*, **217** (1968), 463].

Polyribosomes of growing bacteria

A new method of extracting polyribosomes using low temperature lysis with lysozyme and EDTA supplemented with chloramphenicol to inhibit protein synthesis has been reported from the Department of Biology, Massachusetts Institute of Technology, Cambridge. The superiority of this method over the existing ones lies in its simplicity, wider applicability and better reproducibility. The method has been successfully applied to gram-positive and gram-negative bacteria and yields a significantly larger percentage of polysomes than previously reported. It prevents the extraction of essentially all the polysomes in largely undergraded state from exponentially growing bacteria and thus makes possible rapid kinetic studies of normal cell polysomes.

To *Esch. coli* cells grown exponentially at 37°C. to 3×10^8-4×10^8/ml., chloramphenicol (100 mg./ml.) is added and the cells are then immediately poured over an equal volume of crushed ice and maintained at 0°C. The cells are then harvested by centrifugation at 5000 *g* for 5 min. After the supernatant is decanted, 0·3 ml.

of a solution containing chloramphenicol (2·0 mg./ml.) is added. The pellet is then resuspended in the same tube at 0°C. with 4·5 ml. of sucrose-salt solution containing sucrose (0·5 mole/litre), tris (hydroxymethyl) aminomethane buffer (0·1 mole/litre, *p*H 8·0) and NaCl (0·1 mole/litre). Then 0·6 ml. of freshly dissolved lysozyme (1·0 mg./ml. in sucrose-salt solution) and 0·4 ml. of 0·14*M* EDTA (*p*H 8·0) is added. Protoplasts are formed after 2 min. at 0°C. followed by the addition of 0·12 ml. of 1*M* $MgSO_4$ to restore the magnesium concentration to $10^{-2}M$. The protoplasts are centrifuged for 5 min. at 5000 *g*, the supernatant is decanted and the inside of the centrifuge tube is wiped dry. To the pellet is added 0·5-1·0 ml. of lysing medium and the protoplasts are then disrupted by uniform dispersion in the lysis medium. The crude lysate is centrifuged for 10 min. at 10000 *g* to sediment the cell debris; the supernatant is carefully removed and analysed by sucrose density gradient centrifugation [*Science, N.Y.*, **158** (1967), 658].

Adenosine-3′,5′-monophosphate as the intracellular mediator of ACTH action on adrenal cortex

Though the involvement of adenosine-3′,5′-monophosphate as intracellular mediator of the action of ACTH on adrenal cortex was proposed as early as 1959 by Haynes [*J. biol. Chem.*, **234** (1959), 1421], very little is known about the sequence of events which lead from the ACTH-induced increase in adrenal cyclic AMP concentration to steroidogenesis. Workers of the Departments of Medicine and Physiology, Vanderbilt University School of Medicine, Nashville, Tannessee, have established certain quantitative and temporal relationships among ACTH, adrenal cyclic AMP concentration and steroidogenesis. According to these studies (i) increases in adrenal cyclic AMP concentration occur before increases in the rate of adrenal steroidogenesis; (ii) increasing doses of ACTH produce increasing concentration of adrenal cyclic AMP as steroidogenesis is progressively stimulated, and adrenal concentrations of cyclic AMP remain elevated, while the rate of steroidogenesis is maintained; (iii) the potency of analogues of ACTH in producing stimulation of adrenal steroidogenesis is reflected in their potency in producing increases in adrenal cyclic AMP concentration; and (iv) ACTH increases cyclic AMP levels not only in adrenal quarters *in vivo* and in intact adrenals *in vivo* but also in adrenal homogenates under conditions which suggest that ACTH is acting to increase adenyl cyclase activity.

They have also demonstrated that at a time when the steroidogenetic response to ACTH is blocked by cycloheximide, an inhibitor of protein synthesis, the adrenal cyclic AMP increase is not inhibited. Thus, cycloheximide and, by inference, the newly synthesized protein thought to be involved in steroidogenesis would appear to act at a site past adenyl cyclase. On the other hand, the observed stimulation of steroidogenesis by reduced NADP, which is not antagonized by cycloheximide, and its lack of effect on cyclic AMP levels have suggested that reduced NADP acts either later than ACTH-induced increase in adrenal cyclic AMP concentrations and protein synthesis, or at a point distinct from the series of reactions by which ACTH stimulates steroidogenesis.

Structural Engineering Research Centre, Roorkee

The main areas of activity of the Centre during 1966-67 as revealed by its annual report for the year were: plain and reinforced concrete; prestressed concrete; shell structures, folded plates and space frames; prefabrication; steel and light metal structures; bridges; structural dynamics; multistoreyed structures; and digital computation.

During the year, a handbook for ultimate strength design of reinforced concrete member was issued. Over 100 labour-saving charts and tables, compiled on a computer, have been included in the handbook; it also included ultimate design procedures useful in engineering design work.

The construction of an experimental lift-slab structure, started in 1965, has been completed. All the design and construction skills required for the large-scale application of this process have been perfected. Because of a revision proposed by the Indian Roads Congress, much of the already finished work in standardizing precast, prestressed bridge girders has been repeated. A designers' handbook incorporating the data obtained on such girders in the span range 7·5-36·0 m. has been prepared.

A major activity of the Centre has been its effort to introduce high strength deformed bars as reinforcement in place of smooth mild steel bars. A design for high strength deformed bars has been developed and it has been taken over by the TISCO for commercial production.

With the computer programmes available at the Centre it has been possible to arrive at the minimum weight design of towers for transmission lines.

RIC Reviews

The Royal Institute of Chemistry has started this new journal which covers infrared and Raman spectra of inorganic compounds; chemistry and the consumer; structure and properties of water; chemistry and the origin of life; inorganic polymers; chemicals in the world economy; hydrogen bonding; and chemical discovery. Two volumes would be published per year, the annual subscription being £ 2.

Macromolecules

This new bimonthly journal has been started by the American Chemical Society. It publishes research papers, short communications and occasional reviews on polymer synthesis; polymerization mechanisms and kinetics; chemical reactions of polymers; characteristics of macromolecules in solution; and bulk properties of polymers. The annual subscription is $ 24.00.

FEBS Letters

The Federation of European Biochemical Societies (FEBS) and North-Holland Publishing Co., Amsterdam, are starting this new journal for the rapid publication of short papers in the fields of biochemistry, biophysics and

molecular biology. The subjects to be covered would be protein chemistry; enzymology, biophysical chemistry; nucleic acids; protein synthesis; biochemical genetics; morphogenesis; cellular biochemistry; metabolism; immunochemistry; and natural products. The subscription per volume would be $ 19.00.

BUILD International

The recently established BUILD Foundation of the Netherlands would start this new journal in September 1968. It would be a research and development journal aimed at bridging the gap between building theory and building practice. Developments in building research in different parts of the world would also be reported. Further information can be obtained from the Managing Editor, Weena 700, Rotterdam, The Netherlands.

Announcements

▪ *A Symposium on Recent Developments in Non-ferrous Metals' Technology* will be held at the National Metallurgical Laboratory, Jamshedpur, towards the end of November 1968. The main topics of discussion at the symposium will be: (1) Present status and future expansion of non-ferrous metals industry in India in relation to the five-year plans and world production trends; (2) Indigenous raw materials for non-ferrous industry, their assessment, preparation and beneficiation, etc.; (3) Pyro-, electro- and hydrometallurgical techniques of production of non-ferrous metals and their alloys in the general context of indigenous raw materials and their resources; (4) Physico-chemical reactions governing the extraction metallurgy of non-ferrous metals and alloys based on theoretical, thermodynamic and thermal efficiency consideration; (5) Reclamation and refining of metal scrap and waste and development of indigenous secondary alloy industry; (6) The role of non-ferrous metals and alloys in engineering industries; (7) Recent technological advances in metal casting including continuous casting technique, special metal working processes including cladding, extrusion, deep drawing, pressing and wire-drawing, etc.; (8) Recent developments in heat treatment of non-ferrous metals and alloys; (9) Recent developments in the technology of ' powder metallurgy ' of non-ferrous metals and alloys; (10) Physical, chemical and corrosion resistance properties of non-ferrous metals and alloys; (11) Physical metallurgy of non-ferrous metals and alloys; and (12) Recent analytical techniques for the estimation of non-ferrous metals and alloys.

Further details can be obtained from Shri P. I. A. Narayan, National Metallurgical Laboratory, Jamshedpur.

▪ *An International Symposium on New Sources of Proteins in Human Nutrition* will be held in Amsterdam during 24-29 November 1968. The discussions at the symposium will relate primarily to new sources of animal and vegetable proteins, food yeasts, synthetic amino acids and petroleum yeasts. Further information can be obtained from the International Commission for Agricultural and Food Industries (CIIA), 18 Avenue de Villars, 75-Paris 7.

▪ *Dr P. N. Raju Oration Award* — The subject of oration for this Rs 1000.00 award for 1968 is ' Research in general practice and its contributions to the advancement of medical knowledge '. Nomination of any eminent medical practitioner with a short biographical sketch and a note on his/her contributions in the field of research may be submitted to the Director-General, Indian Council of Medical Research, Post Box 494, Ansari Nagar, New Delhi, before 31 August 1968.

▪ *The Eighth International Congress on Gerontology* will be held during 24-29 August 1969 in the Sheration Park and Shoreham Hotels in Washington DC and Baltimore respectively. Such congresses, held every three years, are sponsored by the International Association of Gerontology. The congress will include colloquia and short papers on biological, psychological, sociological aspects of ageing and on health problems of an ageing population. Further information can be obtained from the Secretariat, Eighth International Congress on Gerontology, 9650 Rockville Pike, Bethesda, Maryland 20014, USA.

▪ *The Twelfth Indian Standards Convention* organized by the Indian Standards Institution will be held at Bhubaneswar during 15-21 December 1968. The object of the convention is to provide a forum for scientists, technologists, industrialists and others to share their knowledge and experience in selected fields of industrialization. Further details regarding the convention can be obtained from Shri Kavaljit Singh, Organizing Secretary, Twelfth Indian Standards Convention, Manak Bhavan, 9 Bahadur Shah Zafar Marg, New Delhi 1.

▪ *An International Conference on Structure, Solid Mechanics and Engineering Design* will be held in Southampton during 21-25 April 1969. The aim of the conference is to consolidate available knowledge and hypotheses related to the structure of certain engineering materials, the influence of their structure on their behaviour under load and the application of this knowledge in engineering design. The materials to be specially considered are rock systems, granular systems, granular cementitious systems, fibre reinforced systems and ceramics. Further information can be obtained from the Secretariat, M. Te'eni, Department of Civil Engineering, University of Southampton, Southampton SO9 5NH.

▪ *The Twentieth Conference of the Indian Pharmaceutical Congress* will be held at Ahmedabad during 24-26 December 1968. The conference will cover the following fields: (i) Industrial pharmacy and microbiology; (ii) Professional and forensic pharmacy; (iii) Pharmacognosy and phytochemistry; (iv) Pharmacology; (v) Pharmaceuticals (medicinal and analytical); (vi) Ayurvedic and Unani pharmacy; (vii) Hospital pharmacy; and (viii) Educational pharmacy. Further particulars regarding the conference can be obtained from the Hony Secretary, Indian Pharmaceutical Congress Association, 18 Convent Road, Calcutta 14.

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