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# Borderland Problems in Biology and Physics

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## FOREWORD

I WAS tempted to use the title "Biophysics" for this review as more succinctly delimiting the field discussed. But despite the obviously increasing interest in biophysical problems, there does not seem to be clear agreement, even among biophysicists, as to what the term biophysics means.

Historical precedent is, no doubt, partly responsible for this confusion. Many years ago, when few physical methods were applied in biological research and when the effects on organisms of such physical agents as x-rays and ultraviolet light were comparatively unknown, biophysics meant "the physics of living organisms." Meanwhile, times have changed. Physicists who venture to explore problems in biology or biochemistry nowadays are more apt to be concerned with the development and application of new physical methods of experimentation, or with the study of the biologic effects of physical agents, than with observation of the physical principles at work in the living organism. If we broaden the term biophysics to include these newer realms of investigation, redefining it as "physical methods and physical principles applied to biology and biochemistry," then "Biophysics" serves perfectly well as a title for this review.

We may subdivide this field into: I. Application of physical methods to the investigation of biological and biochemical problems; II. Study of the effects of external physical agents on living organisms and biochemical substances; and III. Physical phenomena occurring in living organisms. These three subdivisions are considered separately in the three principal parts of the review.

Biophysics is a particular field of *applied physics*, and a natural approach to it seems, therefore, to be from physics as well as from biology, physiology, or biochemistry. Probably this explains why many of the investigations discussed in what follows were initiated by research investigators trained primarily as physicists.

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# Part I—Application of Physical Methods to the Investigation of Biological and Biochemical Problems

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# Part I. Application of Physical Methods to the Investigation of Biological and Biochemical Problems

RECENT advances in this field have been so rapid that it is necessary to limit extended discussions to selected topics. The following subjects have been chosen for detailed consideration because they deal with methods of broad application or because they are of especial current interest: use of isotopes as tracers in physiological investigations; x-ray studies of biochemical compounds; applications of infra-red, visible, and ultraviolet spectroscopy; applications of the ultracentrifuge; and recent advances in microscopy with particular regard to ultraviolet and electron microscopes. Other matters will be considered more briefly.

## *I. ISOTOPES AS BIOLOGIC TRACERS*

The intermediate history and ultimate fate of substances ingested by organisms, and the synthesis of organic compounds in organisms, are matters of great importance in physiology. For the investigation of such problems, two methods of labeling substances have been developed whereby their location in the tissues and excreta may be determined, and their synthesis into other compounds followed. The first originated with G. Hevesy at the Institute of Theoretical Physics, Copenhagen. It depends on the use of radioactive isotopes to label such substances as lead, bismuth, phosphorus, etc. The second, developed by Schoenheimer and Rittenberg at Columbia, makes use of nonradioactive isotopes, such as deuterium, incorporated into compounds as labels.

The value of both methods lies in the fact that the chemical properties of the isotopes used are essentially identical with those of their more common prototypes. Therefore their biochemical history in the organism may be expected to be typical. The slight chemical differences in isotopes and the possible bearing of these differences on their use as biologic tracers have been considered by Taylor (1)* and Goudsmit (2). Barnett (3) stated that the radioactivity of radioisotope indicators might cause marked physiological effects which would invalidate conclusions from their use. Hevesy (4), Crane (5), Mullins (6) and Matilda Brooks (7) have all presented evidence, however, that in the concentrations in which such indicators are ordinarily employed in tracer experiments, their physiologic effects have little or no significance; though Mullins (8) did find that $Na^{24}$ used as a tracer apparently decreased the rate of exchange of Na ions between cells and media as a result of its radioactivity.** Blüh (114) has recently suggested methods for the investigation of the possible biologic defferentiation between isotopes, and of the radiation effect, by the use of mixtures of radioactive isotopes.

The physical differences of the isotopes enable them to be located and determined quantitatively in tissues, tissue fractions, and excreta.

### A. Radioactive Isotopes

#### 1. Methods employing radioactive isotopes

Hevesy developed the method of radioactive tracers because of a problem suggested by Lord Rutherford: the extraction of Ra D from radio lead. In Hevesy's words (9): "I tried numerous methods of separation without having the slightest success. This was a disheartening result and to make the best of the situation I decided to

* Numbers in parentheses refer to literature cited in the bibliography.

** The literature dealing with the effects of radioactivity on physiological processes will be considered later.

reverse the problem to mix a known amount of pure Ra D, which can be easily obtained from radium emanation, with a known amount of lead salt and to follow up the path of the lead atoms by using Ra D or the lead isotope Th B as an indicator."

Beginning in 1913, in association with Paneth (10), he published an extensive series of chemical investigations involving radioactive indicators.† The method was first extended to biologic problems in 1923 (12).

*General procedure*

The general procedure followed is to introduce the radioactive element into the organism either in ionizable form (for example, as a constituent of a salt, which is the usual practice), or as a non-ionizable substituent of a carbon compound, and to trace the subsequent fate of the element by measuring the radioactivity of the various tissues and excreta (usually after ashing).

One of Hevesy's early studies, on the uptake of lead by plants (12), will serve to illustrate. Plant roots were immersed in solutions of mixtures of lead nitrate and Th B nitrate for one to forty-eight hours. The plants were then ashed, and the quantities of Th B in the ash of various parts determined electroscopically. The quantitative determinations were based on the activity of a known amount of the dried original solution, mixed with inactive ash, as a standard. It was assumed that no selective action took place in favor of the Pb or Th B salts in the plant throughout the processes of transportation and assimilation.

Table I lists radioactive isotopes of the principal elements of biologic interest.‡ Radio-isotopes of carbon, nitrogen, sodium, phosphorus, sulphur, potassium, iron, fluorine, chlorine, bromine, rubidium, iodine, lead and bismuth have been used in biologic investigations. Results of value have been obtained with isotopes having half-lives as short as 21 minutes ($C^{11}$), and as experience is extended and technique improved, even elements of shorter half-life may prove to be useful. In the case of several radio-isotopes, their more general use in biology will probably follow

TABLE I. *Radioactive isotopes used in biological investigations.*

| ISOTOPE | HALF-LIFE | GENERAL TYPES OF BIOLOGIC INVESTIGATIONS THUS FAR REPORTED |
|---|---|---|
| $_6C^{11}$ | 21 min. | Photosynthesis in barley plants. |
| $_7N^{13}$ | 9.9 min. | Fixation of atmospheric N by nonleguminous plants. |
| $_9F^{18}$ | 112 min. | Adsorption of fluorides by dentine and enamel of teeth. |
| $_{11}Na^{24}$ | 14.8 hr. | Circulation of ions in plants; ionic penetration of cells; circulation of Na in humans; excretion of Na under various conditions; estimation of fluid content of tissues. |
| $_{15}P^{32}$ | 14.3 days | Circulation of ions in plants; ionic penetration of cells; molecular disintegration and rejuvenation; accumulation, excretion, and rate of turnover of P compounds; P metabolism in pathologic conditions; intermediate carbohydrate metabolism. |
| $_{16}S^{35}$ | 88 days | Excretion by humans; formation of cysteine *in vivo*. |
| $_{17}Cl^{38}$ | 37 min. | Circulation in humans. |
| $_{19}K^{42}$ | 12.4 hr. | Circulation of ions in plants; ionic penetration of cells; circulation in humans; storage in tissues, absorption from g.i. tract; excretion under various conditions. |
| $_{20}Ca^{49}$ | 2.5 hr. | Absorption and excretion in animals. |
| $_{26}Fe^{59}$ | 47 days | Absorption from g.i. tract; excretion; control of Fe level. |
| $_{35}Br^{80}$<br>$_{35}Br^{82}$ | 4.4 hr.<br>34 hr. | Circulation of ions in plants; circulation in humans. |
| $_{37}Rb^{86}$ | 11 hr.(?) | Ionic penetration of cells. |
| $_{53}I^{128}$ | 25 min. | Circulation in humans; accumulation in thyroid gland. |
| $_{82}Th\ B^{212}$<br>$_{82}Ra\ D^{210}$<br>(Isotopes of Pb) | 10.6 hr.<br>16.5 yr. | Circulation of ions in plants: ionic penetration of cells; estimation of blood volume; chemical analyses; excretion and deposition of Pb in tissues; accumulation in tumor tissue. |
| $_{83}Ra\ E^{210}$<br>(Isotope of Bi) | 5.0 days | Excretion and deposition in tissues; Bi in syphilis therapy; accumulation in tumor tissue. |

† A list of such publications up to 1927 will be found in *Radioaktivitat*, reference 11.

‡ The data in this table have been taken in part from a review by Livingston and Bethe, reference 13, which is a valuable source of information regarding the physical aspects of the subject, and in part from a more recent tabulation by Livingood and Seaborg, reference 81.

the more conclusive establishment of their identity, clarification of their physical properties, etc., by further physical research. See, for example, a recent discussion of radio-isotopes of Ca by Walke *et al.* (170).

*Preparation of radioactive isotopes*

For a discussion of the literature on the preparation and properties of various radioactive isotopes, one is referred to the excellent review by Livingston and Bethe (13). Here only a few preparations of particular interest in biophysics will be discussed.

The most commonly used indicator, radioactive phosphorus ($P^{32}$), may be prepared by neutron bombardment of chlorine, sulphur, or phosphorus according to the following equations:

$$_{17}Cl^{35} + {}_0n^1 \rightarrow {}_{15}P^{32} + {}_2He^4$$
$$_{16}S^{32} + {}_0n^1 \rightarrow {}_{15}P^{32} + {}_1H^1$$
$$_{15}P^{31} + {}_0n^1 \rightarrow {}_{15}P^{32}.$$

In a typical preparation (14), ten liters of $CS_2$ were exposed to neutrons from a mixture of 100 mg Ra and several g Be, and after fourteen days the $CS_2$ was distilled off. The $P^{32}$ may also be removed, during neutron bombardment, by an electric field on copper electrodes immersed in the $CS_2$ (15).

An alternative method of preparation is to bombard sodium or phosphorus with deuterons in a cyclotron (16). The $P^{32}$ used by Chaikoff and co-workers (17) was prepared in this way in the cyclotron of E. O. Lawrence, and similar preparations have been used by other workers. The equation of the preparation from $P^{31}$ is:

$$_{15}P^{31} + {}_1D^2 \rightarrow {}_{15}P^{32} + {}_1H^1.$$

Hertz and co-workers (18) used radioactive iodine ($I^{128}$) prepared by bombarding 600 to 1000 cc of ethyl iodide with neutrons from a radium-beryllium mixture (110 mg Ra), the radioactive iodine being concentrated by a method developed by Roberts and Irvine (19) after d'Agostine.

Many of the isotopes used in biologic studies have been prepared by deuteron bombardment in the cyclotron of the Radiation Laboratory of the University of California. For example, the $K^{42}$ used by Greenberg and co-workers (20) was made by bombarding metallic potassium with deuterons, the equation of the transformation being

$$_{19}K^{41} + {}_1D^2 \rightarrow {}_{19}K^{42} + {}_1H^1.$$

The bombarded potassium was dissolved off the target in an atmosphere of $CO_2$, converted into the chloride by adding HCl and evaporating to dryness, dissolved in water, purified from solution by precipitation as potassium acid tartrate, and reconverted into the chloride.

*Measurement of radioactivity of tissues, excreta, etc.*

In the earlier investigations, the radioactivity of tissues, etc. was measured with ionization chambers and electroscopes (for example, see reference 12). While sensitive electroscopes (21) have been employed which are quite satisfactory for highly active preparations, Geiger-Müller counters have now come into general use as more convenient and sensitive detecting devices.

Small counters are used. Hevesy used one with a mica window of 1.1 sq. cm area. The samples to be measured (quantities less than 200 mg) were placed in an aluminum dish immediately below the window. That employed by Perlman, Ruben and Chaikoff had a wall thickness of 0.1 mm of aluminum, and the samples were wrapped around it after being taken up on blotting papers 3 by 6 cm. Hahn and co-workers (22), for their measurements of the $Fe^{59}$ content of samples, used a counter arranged as a plunger which dipped into a cup containing the solution to be investigated so as to form a film about 9.5 mm thick surrounding the counter. Nearly-saturated solutions of potassium acetate were used as standards of radioactivity, and background counts (3 to 4 per minute) were determined with a water or solvent blank. For measurements of weak samples, they electroplated the iron onto thin metal foil, which was wrapped around the counter. Chagraff (23) has described a counter arrangement with convenient accessories for measuring dry samples, Rachel Franklin (24) has published a description of a counter for measuring the activity of radio-sodium, and Brewer and Bramley (25, 26) have developed a sensitive counter for use in plant physiology studies.

Many other suitable counters and accessory amplifiers have been described in the literature in recent years, and commercial units of appropriate design are now available.

When radioactive isotopes of comparatively short life are employed, allowance must be made for decay in activity. This may be done by preserving a portion of the original specimen and using it as a standard in all measurements, as in Hevesy's experiment described above. If radioactive transformation products having strong radiation are formed, as in the case of Th B (which is converted into Th C) further precautions must be taken to insure that equilibrium conditions have been reached when the measurements are made. The absorption by the ash can be compensated for by adding inactive ash of appropriate composition in proper amount to the standard sample.

*Use of chemical fractionation in conjunction with radioactive tracers*

The activities of ashed tissues and excreta yield data as to the distribution of ingested substances in the organism and its excrement, but do not necessarily answer questions about the synthesis of compounds in the organism. For the latter purpose, chemical methods of fractionation may be required. For example, if it is desired to compare the rate of synthesis of phospholipides in the liver and the brain, one may feed or inject radioactive phosphorus as a phosphate, allow a sufficient time for synthesis in the organism, remove the brain and liver and extract the phospholipides,* and measure the radioactivity of the extracts so obtained. By waiting various lengths of time between the feeding of phosphates and the examination of the tissues, the rapidity of synthesis and loss of phospholipides by the tissues may be determined.

It is evident that many combinations of chemical fractionations, chemical tests, and radioactivity measurements are possible in order to study special problems.

In phosphorus metabolism studies, one finds frequent reference to the "specific activity" of samples. This is the activity of $P^{32}$ (determined from radioactivity measurements) per mg of total P as determined chemically.

*Precision of measurements*

The weights of substances which can be determined quantitatively by the method depend upon: (1) The sensitivity of the radioactivity measurements, (2) the ratios of inactive to active isotopes in the preparations used (which varies, of course, with time, because of radioactive decay), and (3) the fraction of the molecular weight of the compound in question represented by the element whose isotope is employed for labeling. Only the sensitivity of measurements with $P^{32}$ will be discussed, it being the radioisotope which has been most extensively used.

Chaikoff (17) states that it is practicable to measure as little as $10^8$ atoms of $P^{32}$ with his counter. Since a $P^{32}$ atom weighs roughly $5\times10^{-23}$ g, this corresponds to $5\times10^{-15}$ g of $P^{32}$. The ratio of $P^{31}$ to $P^{32}$ in typical preparations has been $10^{10}$ or less. The smallest ratio obtainable at the present time is, according to Chaikoff (17) about $10^3$, using the method of Szilard and Chalmers (28) for preparing radioactive elements from compounds of the element.

If $X$ represents the minimum quantity of $P^{32}$ determinable under the experimental conditions, and $Y$ the minimum quantity of labeled P (i.e., $P^{31}+P^{32}$) determinable, then

$$Y=\left(\frac{P^{31}+P^{32}}{P^{32}}\right)X,$$

or, very closely $Y=(P^{31}/P^{32})X$.

The least quantity of a phosphorus-containing compound which can be determined depends, in addition to the above factors, on the mole fraction of phosphorus in the compound. In sodium orthophosphate $Na_2HPO_4$ (frequently used for injection or feeding), this fraction is about 1:4.6 (for $P^{31}$). In a typical phospholipide, $\alpha$-oleyl-palmityl lecithin, it is approximately 1:25.1. Table II, which shows the least quantitatively determinable amounts of various labeled phosphorus-containing compounds of biologic interest for three ratios of $P^{31}$ to $P^{32}$, has been calculated from the sensitivity of Chaikoff's counter and from the mole fractions of P in the compounds listed. It should be remembered that the original ratio of $P^{31}$ to $P^{32}$ increases throughout the experiment (because of the exponential decay of $P^{32}$) to approximately double the initial value in 14 days.

* Kaplan and Chaikoff, reference 27, have described methods for separating the phospholipides in such investigations.

*Types of radioactive compounds employed*

The usual procedure is to administer the tracer in the form of a salt of the isotope used. The isotope is then ionizable in water solution, and under such conditions the radioactive ions can interchange freely with nonradioactive ions of the same elements and thus become incorporated in other ionizable molecules. They do not, so far as present evidence indicates (29–32), interchange spontaneously with nonradioactive atoms of the same element bound by non-ionizable bonds (e.g., linked to carbon). Stable linkages of the isotopic indicators to molecular structures may, however, be formed by the enzymic processes in cells and tissues.

For some investigations, notably those in which it is desired to trace the fate of an ingested organic compound, it would be advantageous to administer the isotopes as stably-linked atoms of the molecules employed. Consequently, there has been some effort to synthesize such compounds, as for example in the investigations of Friedmann and co-workers (33) on methods of forming organic bromo compounds containing $Br^{82}$, those of Alber (34) on the synthesis of organic compounds containing radioactive sulphur ($S^{35}$) and those of Brezhneva and associates (35, 36) on the introduction of radioactive halides into organic compounds. Synthesis within the organism has also been employed—e.g., by introducing labeled phosphates into an animal and recovering labeled phosphatides from its tissues for use in other experiments.

## 2. Results of experiments with radioactive indicators

*Plant physiology investigations*

Among applications of the method to plant physiology may be mentioned studies of the absorption and circulation of ions, of ionic permeability of cells, and of photosynthesis.

The earliest investigations of the circulation of ions in plants were those of Hevesy (12, 37) who immersed roots of growing beans (*Vicia faba*) in $Pb(NO_3)_2$ solutions labeled with Th B nitrate and found greatest lead (Th B) accumulation in the roots, less in the stems, and least in the leaves. Accumulated lead (evidently dissociable) interchanged readily with Pb and Cu (though not with Cd, Zn, Ba or Na) in salt solutions when the roots were immersed in them. More recent experiments, with maize (38) and sunflowers (39), using $P^{32}$-labeled sodium phosphate solutions, showed that P accumulates as inorganic phosphate, the stems containing more than the leaves. The failure of yeast immersed in labeled phosphate solutions to take up $P^{32}$ was interpreted as indicating that yeast P is mainly organic; or, alternatively, that the yeast cell is impermeable to phosphate ions except when the cell is growing (40). These results should be compared with those of Williams and co-workers (41) who grew *E. coli* in media containing $P^{32}$ and found that both the nucleoprotein and phospholipide fractions of the cells later contained $P^{32}$ in the same ratio as that in the culture medium. Gustafson and Darken (42) found, from experiments with willow, geranium, *Sedum praeltum* and *Bryophyllum calycinum*, that both the xylem and phloem are involved in the upward transport of $P^{32}$ ions. Similar investigations by Stout and Hoagland (43), utilizing K*, Na*, and Br*, as well as P*,* showed that the transport of ions was slow in the bark of geranium and willow plants, but rapid in the xylem. The rate of movement of $P^{32}$ ions into bean seedlings was measured by Biddulph (44). Other investigations of the absorption and transportation of ions in plants have included those of Nishina and Nakayana (45) using $Na^{24}$, those of Overstreet and Broyer (46) using K* and those of Brewer and Bramley (47) using P* and Na*.

TABLE II. *Calculated amounts of P*-containing compounds determinable.*

| SUBSTANCE | LEAST AMOUNT DETECTABLE, MG ($P^{31}/P^{32}$) = $10^3$ | $10^5$ | $10^{10}$ |
|---|---|---|---|
| Phosphorus | $5\times10^{-9}$ | $5\times10^{-7}$ | $5\times10^{-2}$ |
| Sodium orthophosphate, $Na_2HPO_4$ | $2\times10^{-8}$ | $2\times10^{-6}$ | $2\times10^{-1}$ |
| Hexose monophosphate, $C_6H_{11}O_6{-}PO_3H_2$ | $4\times10^{-8}$ | $4\times10^{-6}$ | $4\times10^{-1}$ |
| Adenylic acid | $6\times10^{-8}$ | $6\times10^{-6}$ | $6\times10^{-1}$ |
| Coenzyme 1 (diphosphopyridine nucleotide) | $5\times10^{-8}$ | $5\times10^{-6}$ | $5\times10^{-1}$ |

* The radioactive isotopes of elements are frequently indicated by an asterisk superscript following the symbol of the element, thus: P*, C*, Cl*, etc. This notation will be used from time to time herein.

The permeability of plant cells to ions has been the subject of considerable study, but it is difficult to obtain conclusive results by usual methods. As early as 1929, Lark-Horovitz (48) used Th B-labeled $Pb(NO_3)_2$ in sea water to study the penetration of *Valonia macrophysa* by lead ions. Active lead collected in the cell walls of living cells, and some was found in the protoplasm, but practically none entered the vacuole sap (even after four months). Dead cells, on the other hand, were penetrated readily. This was taken to support Osterhout's view that living cell protoplasm is permeable only to undissociated molecules—not to ions.

Extensive investigations of ionic penetration of plant cells, with K*, Na*, Rb*, and Br* as tracers, have been carried out by Brooks *et al.* (49–55). He concludes that ions penetrate cells by interchange with other ions resulting from metabolic processes, and that their concentration in various parts of the protoplasm is concerned with temporary association with radicals of the protoplasm protein molecules. For example, when KCl penetrates *Valonia* or *Nitella*, the $K^+$ ions apparently combine with H-proteinate and the $Cl^-$ ions with protein-OH in the protoplasm, and then migrate from one protein molecule to another until they reach the vacuole, where $K^+$ ions are exchanged for $H^+$, and $Cl^-$ ions for $HCO_3^-$ (or other anions and cations of the vacuole sap). Mullins and Brooks (55) give quantitative data for the rate of loss of Na*, and K* stored in cells in exchanging with various ions in the suspending medium, and emphasize the necessity of considering metabolic effects as well as the Hofmeister series in explaining rates of ionic exchange (52, 53). These studies are especially interesting because of the difficulties that have heretofore beset workers investigating cellular ionic penetration problems, and because they afford excellent examples of the accuracy and simplicity of the radioactive tracer method. The conclusions of Brooks, *et al.* seem reasonable and convincing. In the light of their work the question of penetrability becomes a matter of dynamic equilibrium involving understandable metabolic processes, and the necessity of postulating mysterious properties for cellular membranes and protoplasm to account for such phenomena disappears.

With regard to photosynthesis, radioactive carbon ($C^{11}$) has been used by Ruben, Hassid and Kamen (56) to study the fixation of $CO_2$ in carbohydrates by barley plants in the light and in the dark. The results suggested the presence of a substance, formed in the light, which caused the synthesis of $CO_2$ into carbohydrates in the dark. Thus the fixation of $CO_2$ can apparently take place as a nonphotochemical process brought about by photochemical products previously found in the cell. Although obviously of a preliminary nature, such researches point the way to possible methods of solving the difficult, and extremely important, problem of the nature of the photosynthetic processes.

Ruben *et al.* (57) also used $N^{13}$ to investigate whether nonleguminous plants are capable of fixing atmospheric N, a matter about which there is at the moment considerable controversy. Their results with barley indicate that this nonlegume does fix nitrogen.

It is evident from the foregoing examples that the radioactive tracer method has proven to be a useful tool in a variety of plant physiology investigations.

### *Animal physiology studies with radioactive isotopes of* Pb *and* Bi

Some of the earliest applications of the radioactive tracer method to animal physiology dealt with the distribution of ingested or injected lead in the tissues, and with the excretion of these substances. Injection of $Pb(OH)_2$ and $Pb(NO_3)_2$ infected with Ra D into rabbits and guinea pigs (12, 58) showed that most of the lead is excreted (47.2 percent in the urine and 52.8 percent in the feces in a typical experiment), the greatest amount of the remainder locating in the kidneys and liver and the least in the blood. With Bi-quinine iodide, Bi-K-Na tartrate, and $Bi(OH)_3$, all infected with Ra E, there was greatest accumulation in the kidneys, blood, and lungs (12,59). Of the above Bi compounds, the comparatively low rate of excretion of $Bi(OH)_3$, indicative of its high retention, led to its use in syphilis treatment (60, 61). The evidence for the accumulation of Bi in the liver seems interesting because of the possibility it suggests of liver damage in syphilis therapy with Bi compounds.

Radioactive indicators have been used to compare the deposition of lead, thorium, and bismuth in neoplastic and normal tissues (9, 109, 110) because of the once-favored use of lead compounds in cancer therapy. No selective deposits in tumor were found for lead or thorium, but bismuth did collect to a greater extent in tumor tissue.

Another early application of Th B was the estimation of blood volume in animals by injecting it mixed with citrated blood (164) and comparing the activity of a blood sample taken later with that of the injected samples.

### *Use of* P* *in the study of molecular decomposition and resynthesis of* P-*containing compounds*

To the physicist, perhaps the most interesting animal physiology studies have been those with $P^{32}$, for these have shown that many organic compounds in animal tissues formerly thought to be comparatively fixed are actually undergoing continual molecular decomposition and resynthesis. The animal organism, is, thus, evidently but a temporary resting place for streams of atoms which flow from ingested materials into all the tissues and then, after varying periods of time, pass out again through the media of the secretions and excreta. If this is true, as it seems to be, we must regard living things as dynamic entities in which every part of the organism, down to the most minute portion of each individual cell, is in a continuous state of molecular and atomic flux—entities devoid of fixed structures, like the iron girders of a building, and which exhibit form only as the temporary expression of equilibrium states in which the flow of atoms and molecules assumes definite (but gradually changing) paths characteristic of each epoch in the organism's career.

Thus, from feeding experiments with sodium phosphate containing $P^{32}$, Hevesy and co-workers (62, 172) concluded that bone is such a dynamic structure, the most rapid uptake of active phosphorous taking place in the epiphyses (63, 64). By injecting radioactive sodium phosphate into frogs, Hevesy and Rebbe (65) found that creatine-phosphoric acid, adenosine phosphate, and hexose phosphate molecules in muscle undergo continuous breakdown and reconstruction, the rapidity of the processes being such that *one day should be sufficient for their practically complete replacement.* Perlman, Ruben and Chaikoff (17), using $Na_2HPO_4$ containing $P^{32}$, showed that *the fat depots act as buffers, phospholipides being constantly added to and taken away from them rather than permanently stored in them.* P* was detected in the teeth of young cats by Hevesy *et al.* (64), a few hours after ingestion, and they calculated from quantitative data on humans that the replacement of one percent of the P atoms of human teeth by P atoms from the food requires about 250 days. According to similar calculations, the average time a P atom remains in a rat is about two months (62) and in a rabbit one month (66).* Data for humans indicate (67) that about one-seventh of the P in feces comes into the digestive tract from the blood after having been in body organs for various lengths of time. Of various rabbit tissues investigated, the greatest rate of exchange occurred in the kidney and muscles and the least in the bones (66).

Such dynamic characteristics have even been demonstrated for brain tissues. Hahn and Hevesy (32) isolated lecithin containing P* from the brains of rats, mice, and rabbits within an hour after injection of labeled phosphates. Most interesting studies of phospholipide metabolism in the brains of rats have been reported by Chaikoff and associates (68, 69). They estimated the rate of phospholipide turnover by measuring the amount of labeled phospholipides in different tissues at various periods after ingestion of labeled sodium phosphate. The rate of turnover in the brain was lower than that in the liver, kidney, or small intestine. It was greater in young animals than in old, decreasing quite rapidly from birth until the weight reached about 50 g, and then much more slowly. In the younger rats, the cord showed higher activity than other parts of the central nervous system, while in the older ones the relative activities of the forebrain, cerebellum, and medulla were as great as, or

* It appears, however, that *average* replacement rates for P atoms in an entire tissue or organ may sometimes be misleading. Thus, Manly *et al.*, reference 163, have published experiments indicating that one must regard bone as made up of a labile portion which exchanges P readily with the blood, and a stable portion which acquires P slowly through increments of new calcification and does not exchange P readily.

greater than, that of the cord. It would be interesting if such data could be obtained for humans and correlated with the development of reflexes, rate of learning, etc.

Such investigations as the above indicate the transient history of atoms and molecules in the organism, and illustrate the necessity of considering living things, in all their aspects, as dynamic entities rather than static structures.

*Other animal physiology investigations with* P*

The variety of physiological problems which have been investigated with the aid of $P^{32}$ is so great that it is difficult to survey them concisely. Since this is not a physiological review, we shall limit ourselves to sketching the general types of problems studied, together with a few typical results. One may roughly divide such investigations into the following categories:

*a. Studies of the relative accumulation of* P *in various tissues at a fixed time after its ingestion or injection, and of its relative excretion in the urine and feces.*—Usually these investigations have been carried out with P*-labeled inorganic phosphates, though Haven and Bale (70) used P*-labeled phospholipides from tissues of animals which had been given labeled phosphates, thus utilizing the animal as a means of sythesizing organic compounds containing P*.

One may express the relative accumulation of P in various tissues in terms of either the total amount per tissue or organ, or the amount per unit weight of tissue. The order will not, in general, be the same, for if an organ weighs enough, its total accumulation of P may be high even though its accumulation per unit weight is low. Cohn and Greenberg (71) found the following decreasing order for *total* P accumulation: bone, muscle, liver, stomach plus small intestine, blood, kidneys, heart, lungs, and brain; and for P *per unit tissue weight:* bone, liver, stomach plus small intestine, heart, kidneys, lungs, blood, muscle, skin and brain. They give data for the rates of deposition and loss of P* by different tissues at various times after ingestion.

Other aspects of the problem of P accumulation, rate of turnover and excretion have been studied by Hevesy *et al.* (62), and by Cook and co-workers (72, 73). Armstrong (74) has demonstrated exchange of P* with P in powdered dentine and enamel *in vitro*. Aten and Hevesy (75) showed that the inorganic phosphorus in goat's milk arises from the rapid utilization of plasma phosphates by the milk glands.

*b. Studies of the comparative rate of turnover and of the origin of organic* P *compounds in various tissues and organs.*—This is essentially an extension of the work already discussed on molecular decomposition and resynthesis of P-containing compounds. Artom and associates (76–78) found the greatest production of P*-containing phospholipides and nucleoproteins in the liver, followed, in order, by intestine, kidney, parenchyma, muscle, and brain and medulla. Chaikoff *et al.* (17, 79, 80) obtained similar results. They also found that egg-laying increased phospholipide activity in the hen's blood, oviduct and ovaries. This last investigation suggests numerous possibilities of utilizing radioactive tracers in studying the influence of factors such as age, external environmental conditions, etc., on metabolic processes.

Despite the importance of nucleic acids and their derivatives in such life processes as reproduction, cellular respiration, etc., little is known about the formation of such substances in the cell. The use of P*-labeled sodium phosphate by Hahn and Hevesy (131) to study the rate of turnover of nucleic acids in animal tissues is therefore important in that it brings a new technique to the elucidation of this problem. All the tissues they examined showed an appreciable turnover of nucleic acid; that in muscle being greatest, that in liver next, and that in brain least. But in no instance was the turnover rate as great as that of phospholipides. They point out, however, that the low rates obtained might be accounted for, in part, by a low rate of penetration of inorganic P into the nuclei of cells. *In vitro*, no appreciable exchange of nucleic acid P with sodium phosphate P* was found.

Other studies of P turnover have included the following: Hahn and Hevesy (82) introduced, into a rabbit, plasma which contained labeled phosphatide molecules, and found that after four hours one-third of the labeled molecules appeared in the liver and lesser amounts in other organs. Hevesy *et al.* (83, 84) concluded that yolk phosphatides of hens' eggs are taken up mainly from

the plasma, being replaced in the plasma largely by phosphatides formed in the liver, and (85) that phosphatides, as well as nucleoproteins, are synthesized in growing chick embryos. Also, by using P*, it was found that the synthesis of blood lecithin following fat ingestion occurs outside the intestinal tract (87).

The rate at which P exchanges for P* has been shown by Bulliard *et al.* (86) to be most rapid in tissues which are undergoing rapid chemical exchanges or which have a high phospholipide content.

*c. Studies of the effects of various biochemical factors on* P *metabolism.*—Chaikoff *et al.* found that cholesterol decreases (88), choline increases (89), and betaine (90), methionine, cystine and cysteine (160) stimulate phospholipide metabolism in the liver. Various other amino acids, etc. (133) were found to have no effect. Ammonium chloride administration increased total phosphorus metabolism, and phospholipide metabolism in the kidney (161).

*d. Phosphorous metabolism in pathologic conditions.*—Of various pathologic conditions, rickets has been the subject of the greatest number of investigations with P*. Dols and co-workers (96–102) concluded that this vitamin D-deficiency disease is characterized either by increased formation or decreased destruction of phospholipides. Cohn and Greenberg (103) obtained evidence that vitamin D increases the uptake of inorganic P* by the skeleton. Manly *et al.* (104, 105) came to similar conclusions, and found the site of increased P* accumulation to be the metaphyses.

With the possibility in mind of utilizing the selective deposition of P* in bone as a source of therapeutic radioactive radiation in leukemia, the accumulation of P* in the bones and teeth of humans suffering from leukemia has been investigated (106) and it is claimed that in using P*, remissions in chronic human leukemia similar to those brought about by x-rays were obtained (107). P* deposition is said to take place at a high rate in the spleen and lymph glands of leukematous mice (174) and in lymphomas (107), apparently at the expense of its deposition in bone and liver, again suggesting the use of P* as a therapeutic agent. The application of radioactive preparations in therapeutic procedures will be discussed in greater detail in a later section.

In studying the phospholipide metabolism of tumors with P*, marked differences were found in different types of neoplastic tissues (108, 159). Since amino-ethyl phosphoric acid was first isolated from malignant tissue, it was thought to be characteristic of malignancy. Studies with P* tracers have indicated, however, that this substance is merely a normal breakdown product of cephalin (167).

Partial removal of the livers of animals has been shown, by the aid of P*, to result in marked disturbances of phospholipide metabolism (166).

*e.* P* *in nutrition studies.*—Since they found that P contained in the normal diet of their animals was less efficiently absorbed from the intestine than was Na phosphate, Hevesy and co-workers (111) suggested that the radioactive tracer method might be used to study the relative digestibility of different foodstuffs.

*f. Studies of intermediate carbohydrate metabolism utilizing* P*.—Many believe that the chief hope for the physiological understanding of such diseases as diabetes and carcinoma lies in the study of carbohydrate metabolism, because of certain abnormalities characteristic of such pathologic conditions. Here, again, the radioactive tracer method is being used. Parnas (112) and Hevesy and associates (113) prepared P*-labeled hexosemonophosphoric acid, hexosediphosphoric acid, phosphoglyceric acid, and adenyltriphosphoric acid, with enzymes in muscle juice and yeast juice, and then studied the transfer of phosphorus molecules in glycolytic (112) and other carbohydrate metabolism processes (29). In the case of adenosine triphosphate, two of the phosphorus atoms are replaced by P* much more readily than the third, less labile one (115). Similar intermediate carbohydrate metabolism studies have been made by Meyerhof (116, 117). The results are as yet largely preliminary, but they give promise of leading to the elucidation of details of the carbohydrate metabolism processes.

### *Permeability of red blood cells to ions and compounds*

Considerable study has been made of the permeability of red blood cells to radioactively-labeled ions and compounds. The corpuscles in

dog's blood are quite permeable to $Na^{24}$ in NaCl according to Cohn and Cohn (118). Hevesy *et al.* (130) found that the Na in corpuscles was replaced by $Na^{24}$ in one day. Similar rapid exchange of P* ions between corpuscles and plasma (119) was found, but there was little exchange of P*-labeled phospholipides (29, 120). Ions of $K^{42}$ (used as $K^{42}Cl$) did not enter corpuscles readily (121, 130), and it was concluded that the bulk of the K ions in blood corpuscles is not replaced during the life of the corpuscles. On the other hand, in 24 hours eight percent of the K ions of muscle cells was replaced by ions from the plasma (121). Tuttle *et al.* (175) have measured P exchange in the white and red corpuscles of leukematous humans.

*Investigations with* $Fe^{59}$

The absorption and transport of Fe* fed to dogs was investigated by Hahn and co-workers (22), who found that the site of absorption was the small intestine, the colon not being concerned in the process. Of great interest is their data showing that the rate of absorption varies with the need (122), being fast in anaemia and slow when the Fe level is adequate. While there was an initial increase in Fe* excretion in the urine following injection of iron as ferrous gluconate (123), the elimination through both urine and feces was so small as to indicate that iron balance is maintained by control of absorption from the intestine rather than by variation in rate of elimination. After absorption from the small intestine into the blood stream, Fe* appears to be transported by way of the plasma to its ultimate destination in the tissues. Fe* appeared in the red cells a few hours after feeding (122, 169). That this could not be explained by simple exchange of Fe* with Fe in the hemoglobin was shown by further experiments which demonstrated that hemoglobin Fe is firmly bound (173). Blood destruction caused an increase in Fe* elimination in the feces.

Fe* metabolism has also been investigated by Austoni and Greenberg (162).

*Chemical studies with radioactive indicators*

The many primarily chemical studies with radioactive indicators cannot be reviewed here, but two papers should be mentioned because of their biologic interest. Ishibashi *et al.* used radioactive tracers to determine the K content of urine, saliva, and clover leaves (124) and the Na content of milk, saliva, and pine needles (125). Their methods illustrate how the sensitivity of certain chemical tests can be increased by the use of radioactive indicators: For example, Th B may be incorporated in a compound which forms an insoluble precipitate with the substance to be determined, and the activity of the precipitate may then be used as a measure of the amount of substance originally present.

*Miscellaneous investigations with other radioactive indicators*

Rates of absorption of K*, Na*, Cl*, Br*, and I* fed to humans were studied by Hamilton (126–128) by measuring the radiation from the hand. Six to fifteen minutes after ingestion, radioactivity due to K* could be detected, while with the other elements, measurable activity appeared in only three to six minutes. The rate of rise of activity caused by Na*, Cl*, and I* was also faster than that due to K*.

$Na^{24}Cl$ injected into the blood stream (129) has been used to estimate the extracellular fluid content of the tissues (compare the use of Th B for estimating blood volume, mentioned above (164)).

Investigations with K* (20, 132) have shown that potassium is rapidly absorbed from the gastro-intestinal tract in fasting animals, and that most of the K* is stored in the tissues (muscle, liver, etc.).

The excretion of Na* and K* by rats fed on low potassium and low sodium diets was investigated by Anderson and Joseph (134) with the interesting conclusion that sodium deficiency resulted in abnormally high excretion of Na* and retention of K*, indicating interference with the mechanism controlling K and Na excretion. The phenomenon resembles that found in adrenalectomized rats. Administration of cortin restored normal rates of excretion. Following adrenalectomy, administration of NaCl restored normal excretion rates (136, 137). Potassium-deficient rats excreted Na* normally but retained K*. Heppel (135) has measured the diffusion of Na* into and out of the muscles of potassium deficient rats.

Hertz and associates (18, 138) used I* in studying thyroid physiology, showing that I* tends to concentrate to a large extent in the thyroid gland. They also used Ca* to investigate the absorption and excretion of Ca in the rat.

Borsook and associates (140) investigated the excretion of S* following ingestion of labeled sodium sulphate by humans, the results appearing to indicate a retardation of excretion by S* exchange with S in the tissue. This isotope has also been employed in following the formation of cysteine *in vivo* (141), it being found that S* fed in methionine appeared later in cysteine, but not S* fed as the sulphate.

The radioactive fluorine isotope $F^{18}$ has been used to show that fluorides are adsorbed on dentine, enamel and bone, and to determine the rates of adsorption (168); investigations which are interesting because the increased fluorine content of sound teeth is, thus far, *the one established chemical characteristic differentiating sound from carious teeth.*

### 3. Conclusions*

The value of the radioactive tracer technique in the study of physiologic problems is obvious from the number and importance of the contributions that have been made by means of it. The chief limitation to its more extensive employment at the present time is the lack of availability of suitable radioactive isotopes to many workers. The comparative advantages of this and the nonradioactive isotope method will be considered at the end of the following section.

In addition to the papers cited in the foregoing, those particularly interested in this field will find useful information in the reviews by Paneth (142, 143), Hevesy (144–147), Hevesy and Paneth (148, 149), Parnas (150), Krogh (151), Lawrence (152, 153), de Vries (154), Erbacher and Philipp (155), Rosenblum (156), Greenberg (157), Aten (158), Channon (139) and Policard (171).

* From October 28 to November 2, 1940, a Conference on Applied Nuclear Physics was held at the Massachusetts Institute of Technology, Cambridge, Massachusetts, under the sponsorship of the American Institute of Physics. It was not possible to include in the above discussion the many papers of biologic importance presented at this meeting. Abstracts of these papers are scheduled to appear in the *Journal of Applied Physics*.

## B. Nonradioactive Isotopes

### 1. Methods employing nonradioactive isotopes

The use of nonradioactive isotopes in physiologic investigations was originally made possible by the development of methods for fractionating "heavy water" and for the isolation of the heavy hydrogen isotope, deuterium, following the spectroscopic demonstration of the existence of this isotope in 1931 (176). Largely through the efforts of Schoenheimer, Rittenberg and associates at Columbia University, the technique of using this isotope as a tracer in biologic problems has been highly developed. With improved methods of preparation, such isotopes as $C^{13}$, $N^{15}$, $O^{18}$, $S^{34}$ etc. have also become available in recent years, but of these only $N^{15}$ has been extensively employed in biologic studies.

In general, the scheme of using nonradioactive isotopes as tracers is similar to that employed with radioactive isotopes. The tracer is incorporated into a compound which is fed or injected into the organism, and subsequently the abundance of the isotopic tracer in tissues, excreta, etc., is determined. Alternatively, the organism may be maintained in an environment rich in the isotope (e.g., in heavy water, or in an atmosphere of high $O^{18}$ content), and a determination made later of the isotopic content of the tissues, or of compounds isolated therefrom. In particular, however, the methods of preparing the isotopes, of incorporating them into compounds, and of determining their relative abundance differ from those employed with radioactive tracers.

*Preparation of nonradioactive tracer isotopes*

Numerous methods of concentrating nonradioactive isotopes have been attempted, with varying degrees of success—methods based on difference in diffusion rates, fractional distillation, chemical exchange reactions, solubility differences in two adjacent phases, high speed centrifugation, etc. The hydrogen isotope $D^2$ was early concentrated by fractional distillation (177), but the preparation of its oxide, heavy water, by electrolysis (178, 179) has displaced other methods because it permits a high degree of concentration with comparative ease and simplicity. If heavy water so prepared is to be used for biological experiments, it must be freed

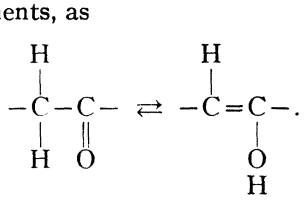
carefully from impurities (180). To obtain $D^2$ from heavy water, it is merely necessary to decompose it by electrolysis. Traps at $-80°C$ serve to freeze out water carried over with the $D^2$, and the dry gas may be collected over mercury for subsequent use (181).

Concentration of $C^{13}$ (182–185), $N^{15}$ (186–189), $O^{18}$ (190), etc., requires more elaborate procedures, and this is one of the factors which has thus far limited their use in biology.

The general problem of separation of isotopes has been reviewed by Urey (191) and Hevesy and Paneth (148), among others, and Taylor (192) has published a brief review of newer methods of separation. Bramley and Brewer (193, 194) have described interesting variations of the thermal diffusion method of Clusius (195).

*Methods of labeling compounds*

One of the important advantages of $D^2$ as a tracer is its ability to be linked stably by a non-ionizable bond to the carbon skeleton of a compound which it is desired to label, so that it remains as a permanent marker of the particular carbon atom in question throughout any but the most drastic chemical transformations. The advantage of such stable labeling in following the physiologic fate of compounds has already been pointed out. It is necessary for this purpose, of course, that the $D^2$ be bonded directly to a carbon atom (if it were introduced, for example, as a part of an OH group, i.e., O—D, it would be labile). The effect of certain neighboring groups in labilizing $D^2$ linked to carbon must also be avoided; e.g., groups which permit tautomeric rearrangements, as



One convenient method of stably linking $D^2$ to carbon is to use $D^2$ to saturate an unsaturated organic compound (196). For example, if crotonic acid is treated with $D^2$, and platinum, palladium, or nickel is used as a catalyst, the C=C bond becomes saturated by addition of $D^2$, and $\alpha$, $\beta$-deutero butyric acid results. This has one-fourth of its hydrogen as $D^2$. Among tagged compounds which have been made in this way are: propionic acid (197), butyric acid (197, 198), caproic acid (197), stearic acid (199), leucine and valine (200), homocystine and methionine (201), ornithine (202) and coprostane (203).

Hydrogen attached to carbon can be replaced with $D^2$ by the use of certain catalysts, such as active platinum (204–209), and $D_2SO_4$ (210–212). Heating alpha-amino acids at 100–170° in strongly acid or alkaline heavy water results in the introduction of $D^2$ in stable C–D linkage (213). Other methods have been discussed by Erlenmeyer (214).

Finally, it is possible to isolate deutero compounds from organisms maintained on water containing a high percentage of $D_2O$, or to feed one deutero compound (such as deutero-oleic acid) to an organism in order for it to be converted into another (such as deutero-stearic acid) *in vivo*, the desired conversion compound being recovered from the organism (215).

In the case of the nitrogen isotope, $N^{15}$, labeled ammonia has been used in the synthesis of various compounds. Labeled glycine, alanine, phenylalanine, tyrosine, norleucine, leucine, glutamic acid, aspartic acid (216), ornithine (202), $\alpha$-amino-$\gamma$-phenyl-barbituric acid (217), sarcosine, creatine, and creatinine (218) have been synthesized in this way. *In vivo* formation of $N^{15}$-labeled compounds is a theoretically possible method of preparation, but low yields prevent its practical use at present.

*Determination of isotopic content of tissues, etc.*

*a. Preparation and purification of combustion water for $D^2$ determinations.*—Determinations of the deuterium content of tissues, etc., may be made conveniently by refractive index or density measurements of the combustion water. For example, if a $D^2$-labeled fat is fed and it is desired to determine what percentage of this fat has located in particular tissues, the tissues are burned in a combustion furnace similar to that used for ordinary organic analysis, and the combustion water is isolated. The water must then be freed carefully from impurities—often a lengthy and tedious process. Appropriate methods of purifications have been described by various authors (181, 219, 220).

In a typical arrangement (181), vapor from the combusted material was carried through two tubes filled with copper oxide and heated to 750°C. Enough tissue was combusted to form 100 to 300 mg of water, and this was collected in a trap at −80°C. It was then introduced with a small amount of dry chromium trioxide into the first of a series of five traps, forming a closed system, and sealed off. It was heated, frozen, and then sublimed into the second trap, which contained a small amount of dry alkaline permanganate. After this, it was sublimed from trap to trap until it collected in the last one. In the end, a few drops of purified water were obtained for determination of the heavy water content. The entire procedure was repeated until the same values for density, or index of refraction, were obtained in two successive purifications. More recently, Keston, Rittenberg and Schoenheimer (220) have reported that agreement of the values of $D_2O$ content determined by both index of refraction and density measurements is a reliable criterion of sufficient purification.

*b. Density determination of combustion water.*—The ratio of the densities of $D_2O$ and $H_2O$ at 25°C is about 1.107 [Lewis and MacDonald (221): 1.056; Taylor and Selwood (222): 1.1079; Tronstad *et al.* (223): 1.1074; Johnston (224): 1.10763].

Pycnometers can be used for the density determinations, but they require a considerable quantity of water (about 5 cc), and the method is not as rapid as some others. The maximum accuracy is about one part in a million, corresponding to about 0.001 percent $D_2O$ in $H_2O$.

The submerged float method is based on adjusting the temperature (225), hydrostatic pressure (226), or pull of an external magnetic field on a piece of iron in the float (227), until floating equilibrium is attained (i.e., until the submerged float neither rises nor falls). The device may be calibrated with solutions of known density (the temperature-density relation with temperature operated floats is nearly linear), or with water containing known fractions of $D_2O$. Schoenheimer *et al.* (220) claim an accuracy of 0.005 percent in $D_2O/H_2O$ determinations with a pressure-operated float, but they state that the method is tedious.

The falling-drop method, developed by Barbour and Hamilton (228, 229), is convenient and accurate, and requires but a small quantity of water. In this method, one determines the time required for a drop of predetermined size of the liquid under investigation to fall through a given distance in an immiscible fluid of slightly less density. In the original device of Barbour and Hamilton, a 0.01-cc drop was timed in a fall of 30 cm through a mixture of xylene and bromobenzene. By varying the proportions of this mixture, the specific gravity of the medium could be adjusted. The device was calibrated with drops of $K_2SO_4$ solution of known density. The method was first applied to $D_2O$ determinations by Vogt and Hamilton (230). Schoenheimer *et al.* (220) used a 0.007-cc drop, which was allowed to fall through *o*-fluorotoluene instead of a xylene-bromobenzene mixture in order to avoid changes in the relative concentration of xylene and bromobenzene resulting from evaporation. They claim an accuracy of one part per million in density measurements.

Linderstrøm-Lang *et al.* (231) have described a "gradient tube" method in which a drop of the water, the density of which is to be determined, is introduced into a vertical glass tube containing a mixture of kerosene and bromobenzene which varies linearly in density with height. The drop (0.001 cc) comes to rest at a point at which its density is equal to that of the mixture.

*c. Refractive index measurements of combustion water.*—The difference in $n^{D}_{20}$ between $H_2O$ and $D_2O$ is about 0.00462 (222), and in mixtures of the two, the refractive index has been shown to be a linear function of the mole fraction (232). With an interferometer for the refractive index measurements (233–236), the accuracy of determination of $D_2O$ in $H_2O$ is about 0.02 percent, and a sample of about 0.4 cc is required (220).

*d. Thermal conductivity method for estimating* $D^2$.—The difference in thermal conductivity between $H^1$ and $D^2$ has been made use of in several gauges for the determination of the mole fraction of $D^2$ in mixtures of the two isotopes (237–240). Samples as small as 0.01 g can be used, but the construction and operation of the equipment is naturally more complicated than of that for density determinations.

*e. Mass-spectrograph method for estimating isotopic tracers.*—Where suitable equipment is available, the mass spectrograph serves as an excellent and precise method for isotope determinations. It is the only method being used for isotopes other than $D^2$. Schoenheimer and associates (241) have described a special mass spectrograph for $N^{15}$ determinations, based on designs by Bleakney (242, 243), which is, of course, equally applicable to $D^2$, $O^{18}$, etc. The ions are produced by impact of electrons from a hot filament with the molecules of the gas, and the ion current for different magnetic field strengths (deflecting different isotopes through the exit slit) is measured with a vacuum tube voltmeter. They state that 0.01 percent $N^{15}$ can be determined with this instrument in mixtures of the nitrogen isotopes. For $N^{15}$ determinations from organic material, the nitrogen is liberated as ammonia by the Kjeldahl procedure and then is converted into gaseous nitrogen by treatment with alkaline sodium hypobromite, the freed gas then being analyzed in the mass spectrograph (241).

*Precision of measurements*

It is interesting to make some rough calculations as to the least quantities of tagged compounds which can be determined by the nonradioactive isotope method. In the case of $D^2$-labeled butyric acid, for example (the preparation of which has been discussed) 25 percent of the $H^1$ atoms are substituted by $D^2$. This is usually expressed by saying that the tagged butyric acid "contains 25 atom percent $D^2$." If butyric acid were isolated, combusted, and a determination of the percent of tagged butyric acid made by density measurements, about 0.3 cc of water would be required, which would correspond roughly to 0.3 g of butyric acid. If the density determinations had an accuracy of one part in a million, about 0.001 percent heavy water could be determined in the sample, after purification. Since the tagged fatty acid contains 25 atom percent $D^2$, the density-determinations would show the presence of 0.004 percent tagged butyric acid, or roughly 0.012 mg. Comparing this value with the amounts of hexose monophosphate, adenylic acid, and coenzyme detectable by the radioactive tracer method with a $P^{31}/P^{32}$ ratio of $10^{10}$ (last column, Table II) one sees that the accuracies are of about the same order of magnitude.

These rough calculations do not take into account errors due to impurities, or the natural abundance of the heavy isotope in unlabeled butyric acid (the natural abundance of $D^2$ in unlabeled compounds is about 0.02 atom percent; that of $N^{15}$ about 0.37 atom percent).

*Biochemical characteristics of isotopes, exchange reactions, etc.*

In using any element as a biologic tracer, consideration must be given to the possibility that its characteristics may cause abnormal physiologic effects. Thus, when hydrogens are replaced by halogens as tracers, marked changes in chemical properties of the labeled compounds result, and this would be expected to influence the physiologic history of the compounds. Possible objections from this standpoint to the use of radioactive tracers have already been considered. In the case of nonradioactive isotope tracers, the differences between the isotopes are so slight, and the difficulties of separating them by any but the most heroic means so great, that it would seem that such objections could be dismissed at once with regard to all except possibly deuterium. In the case of $H^1$ and $D^2$, the two to one ratio of atomic weights might conceivably introduce complications.

The strongest argument that the double atomic weight of $D^2$ is not a legitimate objection to its use as a tracer arises from the apparent inability of organisms to discriminate between $H^1$ and $D^2$ in their ordinary physiologic processes. Thus, the abundance of $D^2$ in naturally-occurring water and in various naturally-occurring organic compounds seems to be about the same (244–247).* Likewise, when animals have been maintained on water rich in $D^2$, a corresponding abundance of $D^2$ has been found in their various organs, blood and urine (248). The abundance of $N^{15}$ in various natural amino acids has been shown to be about the same as that in atmospheric N (249). On the other hand, in the case of the carbon isotope $C^{13}$,

* There is some evidence for a slight biological differentiation between the hydrogen isotopes. This evidence is discussed in a subsequent section on the biological effects of heavy water.

variations as great as five percent in the $C^{12}/C^{13}$ ratio in different compounds have been reported (250), $C^{12}$ being apparently slightly concentrated in plants and $C^{13}$ in limestone.

Despite evidence regarding the nearly identical biological activities of the hydrogen isotopes, it is well known that $D_2O$ in high concentrations is toxic for organisms, and that certain biochemical processes take place at slightly different rates in $D_2O$ or with $D^2$-labeled compounds.** The proportion of $D^2$ used as a tracer should, therefore, be kept within reasonable limits, an entirely practicable procedure in view of the lack of toxicity of $D_2O$ in concentrations of the order of ten percent or less (251).

Finally, as already pointed out, if erroneous interpretations are to be avoided, consideration must be given to the stability of the linkages by which $D^2$ is incorporated in the compound as a tracer.†

## 2. Results of experiments with nonradioactive indicators

*Studies of fatty acid metabolism using* $D^2$

Deuterium is especially valuable as a tracer for fatty acids because of the ease with which it can be stably linked to C in such compounds by the methods already discussed.

When $D^2$-labeled long-chain fatty acids are fed to animals, they are deposited quickly in the fatty tissues and in the fats of the internal organs (252–255), the highest concentration being found in the liver. Even when the feeding level and energy requirements are such that fats must be burned as rapidly as they are fed, there is similar deposition of the ingested fats (252), indicating the action of the fatty tissues as buffers between incoming and outgoing fat supply instead of as permanent storage places. These general conclusions are in agreement with those from experiments with $P^{32}$ previously discussed. Again the dynamic character of the components of organisms is emphasized.

When short-chain fatty acids, such as deutero-butyric and deutero-caproic acids, are fed, they do not appear to be deposited in the fatty tissues but rather to be burned directly, for their $D^2$ is all found in the tissue fluids (197).

Exceedingly interesting investigations with $D^2$ tracers have demonstrated extensive biochemical transformations—degeneration, regeneration, transformation into other compounds, etc.—among fat substances. Thus, fatty acids have been shown to be converted into phospholipides (256). The isolation of deutero-oleic acid from mice fed deutero-stearic acid (259) has furnished evidence that fatty acids are desaturated in the living organism; and, more recently, palmitic acid has been shown to be desaturated to palmityloleic acid (257). The reverse process, saturation of unsaturated acids, was demonstrated by feeding deutero-oleic acid (formed *in vivo* from deutero-stearic acid) and isolating the saturated deutero-acids from the animals (215). Both shortening and lengthening of the carbon chain have been shown to take place *in vivo*. Deutero-stearic acid is partially degraded to deutero-palmitic acid (two carbons shorter) (258). Deutero-palmitic acid is likewise partially degraded to shorter-chain acids, and partially elongated to form deutero-stearic acid (259). Hence, the three principal constituents of animal fats—palmitic, stearic and oleic acids—appear to be interconvertible *in vivo* as a part of the dynamic life processes.

Many other studies of fat metabolism with the $D^2$ tracer can be mentioned but briefly. The source of liver fats has been investigated, and has been shown to vary with the conditions under which the animals are maintained, usually being principally the fat depots (255). Cetyl alcohol ($C_{16}H_{34}O$) and octadecyl alcohol ($C_{18}H_{18}O$) have been shown to arise *in vivo* from fatty acids (260). Fats and fatty acids of animals maintained on a high percentage of heavy water have been found to contain stably-bound deuterium (261–263), which has been interpreted as indicating their synthesis in the animals. When linoleic and linolenic acids were isolated from animals maintained on heavy water, however, they did not contain appreciable $D^2$ (264), indicating that these acids arise only from the diet. A study has also been made by this method of the rates of regeneration of various fatty acids *in vivo* (264).

** These matters are discussed in a subsequent section.

† For a discussion of the literature regarding the influence of adjacent polar groups in labilizing C–D linkages, chemical exchange reactions, etc., in relation to the use of $D^2$ as a tracer, one is referred to an excellent review by Schoenheimer and Rittenberg, reference 251.

*Sterol studies with* $D^2$

Cholesterol takes up $D^2$ from the body fluids of adult animals rich in heavy water (263). The large amount of $D^2$ found in such cholesterol is interpreted as indicating its synthesis from smaller molecular units already containing $D^2$ (e.g., intermediates of fat or carbohydrate metabolism). The half-time of regeneration of cholesterol is estimated at 15–25 days. The mechanisms of the formation of coprosterol and bile acids have also been investigated in this way (203,265,266).

*Carbohydrate studies with* $D^2$

The use of $D^2$ to label carbohydrates involves the difficulty that the bonds formed are labile and subject to exchange reactions. It is possible, however, to take advantage of this lability by maintaining animals on heavy water and assuming that interchange of $H^1$ and $D^2$ in their sugars takes place, as in the studies, described above, of fatty-acid synthesis by animals maintained on heavy water (it is assumed that such synthesis took place from carbohydrates labeled by spontaneous exchange reactions in the organism). Ussing (267) examined the $D^2$ content of the glycogen isolated from animals maintained on heavy water and fed large amounts of monohexoses. He concluded that the $D^2$ content of the glycogen was greater than could be accounted for by simple coupling of monohexoses which had taken up $D^2$, and that there must therefore be a degradation of such molecules into smaller ones prior to synthesis.

*Investigations with* $D^2$ *in lower plants*

When algae (268–270), fungi (271), or yeast (272) are shaken in heavy water, there is an exchange of $D^2$ with $H^1$ in the tissues of the organisms, as shown by measurement of the $D^2$ content of the tissue fractions. If the same organisms are *grown* in heavy water, the $D^2$ content of their tissue is found to be materially higher, which is interpreted as indicating the synthesis by the organisms of compounds containing $D^2$. Fat synthesis in microorganisms has been studied by similar methods (273).

*Studies of protein and amino acid metabolism with* $D^2$

When rats are given heavy water to drink, $D^2$ is found in the body proteins (248, 274–277) and various amino acids (278). Ussing (279) fed a $D^2$-labeled casein hydrolysate to a rat and found, after three days, indications that ten percent of the liver protein and about twenty-five percent of the muscle protein were newly formed from the diet. Tyrosine is continously liberated from proteins and replaced by tyrosine ingested in the diet or formed *in vivo* from phenylalanine, etc., according to investigations of Moss and Schoenheimer (280) in which $D^2$-labeled *dl*-phenylalanine was fed to rats.

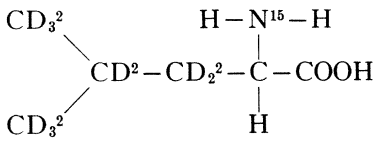
*Chemical investigations with* $D^2$

It is not possible to discuss the many applications of $D^2$ to chemical investigations, but one technique developed independently by Rittenberg *et al.* (281) and by Ussing (282) should be mentioned because of its biochemical usefulness. Ussing utilized $D^2$-labeled leucine mixed with hemoglobin in known quantities to estimate the leucine content of the hemoglobin. Leucine was isolated from the hydrolysate. From the density of the combustion water of the extract and that of the labeled leucine, the ratio of labeled to unlabeled leucine in the extract was determined. If one assumes this ratio to be the same as the ratio of labeled to unlabeled leucine in the original mixture, and knows the quantity of labeled leucine added to the original mixture, the leucine content of the hemoglobin can be calculated from: $L=(l/l_0)L_0$, where "$L$" is the quantity of unlabeled leucine originally present in the hemoglobin, "$L_0$," the quantity of labeled leucine added to the hemoglobin and $(l/l_0)$ the ratio of unlabeled to labeled leucine in the isolated leucine sample. The method has been extended to the estimation of other amino acids (283). Of particular interest is its application to the determination of *d* (−) glutamic acid in malignant tumor tissue (284). The announcement by Kögl *et al.* (285) that this amino acid (not found in normal tissue) is present in tumor tissue aroused considerable excitement because it was the first *qualitative* biochemical difference (as distinguished from *quantitative* differences) to be found between

normal and malignant tissues. Kögl's claim has been disputed. The $D^2$ investigations (284) failed to show appreciable quantities of *d* (−) glutamic acid in tumor tissue, substantiating Kögl's opponents.

*Experiments with* $N^{15}$

The nitrogen isotope $N^{15}$ has been of particularly important service in the study of protein metabolism. A brief review of early investigations has been published by Schoenheimer *et al.* (286). By means of labeled ammonium salts, the utilization of ammonia in the synthesis of various amino acids and of proteins *in vivo* has been demonstrated (287, 288). The synthesis of amino acids into proteins has been studied, with labeled *dl*-tyrosine (289), *l* (−)-leucine (290), and glycine (291). In the case of *l* (−)-leucine, the carbon chain was labeled with $D^2$ and the amino group with $N^{15}$, as shown below. After feeding, approxi-



mately half of the labeled nitrogen of these amino acids was recovered from proteins of the animals.

By using $D^2$- and $N^{15}$-labeled leucine (290) and $D^2$-labeled protein hydrolysates (279), it was shown that fairly rapid regeneration of proteins in muscle and liver takes place, and that there is considerable replacement of protein amino acids by corresponding amino acids from the diet. However, other evidence shows that dietary amino acids incorporated into proteins do not remain altogether intact as such, for $N^{15}$ has been isolated from amino acids other than those fed (289–291). Investigations of histidine from animals given $N^{15}$-labeled ammonium salts, etc., have indicated that its α-amino group is capable of undergoing continual deamination and amination, and that its $N^{14}$ can be replaced by $N^{15}$ in this way (292). Similar nitrogen interchange has been demonstrated for *l* (−)-leucine (290), *d* (+)-leucine (293), glycine (291), and for *d*(−)-phenyl-amino-barbituric acid while undergoing biological conversion to the acetyl derivative of its *l* (+) form (294).

That such nitrogen interchange is a result of chemical processes (e.g., amination and deamination) rather than a mere exchange of labile atoms is indicated by experiments in which such interchange failed to take place *in vitro* in water solutions at 100°C (217). Evidence that the mechanism of the interchange is generally one of deamination and subsequent amination, and further indications of the high chemical activity of tissue proteins has been presented by Schoenheimer *et al.* (295). When $N^{15}$ labeled sarcosine is fed, however, it is apparently demethylated directly to form glycine, without deamination (296).

Similar rapid nitrogen assimilation and interchange with amino acids and proteins has been demonstrated for growing tobacco and buckwheat plants, by using N*-labeled ammonium chloride (297, 298).

Such studies as the above tend to emphasize once more the dynamic state of compounds within the living organism.

Two other investigations with $N^{15}$ may be mentioned: $N^{15}$-labeled glycine has been used to study the formation of hippuric acid (299), and $N^{15}$- and $D^2$-labeled *d* (+)-leucine has been used to demonstrate the inversion of this amino acid to the *l* (−) form *in vivo* (293).

*Investigations with* $O^{18}$ *and* $C^{13}$

The use of $O^{18}$ as a tracer is limited by the fact that $O_2$ as incorporated in organic compounds is not stably linked. However, useful biological studies with $O^{18}$ are possible. Thus Day and Sheel (300) used $O^{18}$-enriched air to demonstrate that inhaled $O_2$ enters directly into carbon oxidation and is exhaled as $CO_2$, and Aten and Hevesy (301) used $O^{18}$-labeled sodium sulphate to determine whether sulphate ions exchange their $O_2$ *in vivo*, the conclusion being negative.

One preliminary paper (302) has reported studies of fixation of $CO_2$ by bacteria, with $C^{13}$ as a tracer.

### 3. Conclusions

In conclusion, let us consider the comparative advantages and disadvantages of nonradioactive and radioactive tracers. The usefulness of both kinds of tracers in physiologic investigations has been established conclusively. More radioactive

than nonradioactive isotopes are at present available in sufficient quantity to be used as tracers. On the other hand, the radio-elements used as tracers are not adapted to stable labeling of carbon skeletons except in the case of $C^{11}$,which has a very short half-life (21 min.). In contrast, as we have seen, deuterium lends itself admirably to the fairly permanent labeling of carbon skeletons, and $N^{15}$ serves as effectively for stably labeling amino groups, etc. Radioactive tracers are easily assayed quantitatively, even in the intact organism, while nonradioactive tracers require elaborate procedures to isolate and purify them, prior to their assay, and to determine them in the purified preparations. However, decay of radioactivity complicates the assay of radioactive isotopes. Finally, objections to the use of isotopes on the grounds of differences in biochemical or biological properties are probably more valid in the case of deuterium, the most widely used of the nonradioactive tracers, than in the case of the other tracer isotopes.

For detailed discussions of the preparation and properties of deuterium and heavy water, one is referred to the book by Farkas (303) and the excellent review in this journal by Urey and Teal (304). The review by Schoenheimer and Rittenberg already referred to (251), together with an earlier and briefer review by the same authors (305) gives a comprehensive survey of the results of physiologic studies with nonradioactive tracers, and similar information will be found in reviews by Channon on fat metabolism (139), and by Lewis and Garner on protein metabolism (306).

## *II. X-RAY STUDIES OF THE STRUCTURE OF BIOCHEMICAL SUBSTANCES, TISSUES, ETC.*

What the isotopic tracer method has contributed in establishing the dynamic characteristics of the molecular constituents of protoplasm has been paralleled by the contribution of x-ray diffraction techniques in determining the patterns into which such molecules aggregate in building up the foundations of the grosser structure of the organism.

A living organism may be likened to a tapestry which is undergoing continual wear and repair, the design being maintained approximately the same throughout the tapestry's life. Isotopic tracer studies have shown that, unlike a tapestry (which is repaired only when noticeable damage occurs and then only in that particular spot) the whole fabric of organisms is in a continual state of being torn down and rebuilt, such processes evidently being controlled by various equilibrium conditions. It was emphasized that many parts of the living being are more "alive" than we had hitherto thought them to be. Thus one aspect of living things which distinguishes them from the dead is that practically all of their parts seem to be in dynamically steady states, capable of being adapted to marked changes in conditions.

The design of a tapestry is woven in thread of different colors, and the ultimate qualities of the fabric, such as its softness, ability to withstand wear, etc., are determined by the characteristics of the thread as well as those of the weaving. One can readily see with one's eyes alone the general design and pattern of a tapestry and the gross structure of the threads of which it is woven (its gross anatomy) but one must resort to the microscope to see the fine structure of the threads (its microanatomy or histology). Even then, one does not find an explanation of the qualities of the fibers which constitute the threads—their elasticity, pliability, tensile strength, etc.—qualities which are reflected in the characteristics of the tapestry. For such explanations, one must discover the patterns and arrays of molecules and submolecular units within the fibers of the threads—their manner of packing, the changes in their packing under strain, etc. It is knowledge of these things which x-ray crystallography reveals to us.

Observation of the structure of the living organism is quite analogous, except that the situation is usually complicated by the necessity of killing the organism, and one does not always know what changes are brought about by death. Gross structure (anatomy) may be observed with the eye alone, microstructure (histology) with the aid of the microscope, but for observation of the states of aggregation of the molecules and

submolecular units, which govern the qualities of the living fabric, one needs to resort again to x-ray crystallographic methods. It is only in recent years that extensive application of x-ray methods have been made to such problems, and the extent and importance of the many contributions in this field is, therefore, especially impressive. Interestingly enough, it was partly through studies of textiles by x-ray diffraction methods that the application of such methods to many biological and biochemical problems developed.

## 1. Methods

The methods used for x-ray crystallographic analysis of substances of biochemical interest are essentially the same as those employed for inorganic materials, with these exceptions: (1) Since the spacing between molecules or submolecular units is the usual object of the analysis rather than the spacing between atoms, more-sharply-defined beams or longer wave-lengths are used; (2) because of the low order of symmetry of many of the materials investigated, the interpretation of the spectra frequently presents a problem somewhat different from that encountered in the investigation of large crystals.

There are a few instances of substances of biologic interest (e.g., certain organic compounds) in which large single crystals are available and the Laue, Bragg, or oscillating crystal methods of analysis may be employed. Usually, however, the substance is either in the form of a crystalline powder, a fiber, or a more or less random aggregate, and the Hull-Debye-Scherrer powder method or monochromatic pinhole method must be employed. In the case of long-chain hydrocarbons it is possible to obtain films of multiple molecular layers which act like built-up crystals, and to determine, from the patterns produced by such films, the molecular lengths and side spacings. If the film is formed on a flat plate, the oscillating crystal method is used; whereas if it is permitted to form on a highly curved surface, such as that of a drop of mercury, and the x-rays are directed against this surface tangentially, they strike the surface at various angles, and oscillation is not necessary. In the case of substances of low melting point, a drop of the material itself may be used, sufficient cooling being permitted to provide only a thin solid surface on the drop. Fibers or stretched materials are conveniently examined by the monochromatic pinhole method, the specimen usually being orientated with the fiber axis perpendicular to the x-ray beam.

### *Technique and equipment*

The tubes and accessory equipment may be of conventional design if they provide radiation of sufficiently long wave-length and intensity (see Clark's book (307) for a general description of such equipment).

Copper targets ($K\alpha = 1.539$A) are most frequently employed. For very large interplanar distances, the longer characteristic radiation of iron ($K\alpha = 1.935$A), chromium ($K\alpha = 2.287$A), aluminum ($K\alpha = 8.31$A) or magnesium ($K\alpha = 9.86$A) may be used, since the spacings in the diffraction pattern are less for large interplanar distances in the specimen but greater for longer wave-lengths of radiation ($n\lambda = 2d \sin \theta$). From the results of Clark *et al.* (308) it appears to be practicable to measure interplanar distances as great as 400A with copper $K\alpha$-radiation and the fiber method, if a sufficiently small pinhole (0.005 inch) and a sufficiently large specimen to film distance (20 to 30 cm instead of the usual 5 cm) are employed, and the central beam is carefully shielded from the plate with a small lead bead. For still larger interplanar distances, they have used a magnesium target tube with camera combined in the same unit (309). The evaluation of large interplanar spacings from measurements of the intensity distribution in the central spot has been described by Hosemann (310, 311). Spacings upwards of 500A have been measured with copper radiation by Bernal and Fankuchen (414, 415) in the case of virus proteins by using well-defined beams.

In order to shorten times of exposure, tubes producing high intensities of x-rays may be used. Astbury has employed a tube with spinning, water-cooled target which permits the photographing of organic materials in comparatively short time (312). Its design is based on one built by Müller (313, 314). Clark has made use of a modified Ott (315) tube in which the specimen is quite close to the target and the intensity correspondingly high.

Filtered radiation is usually employed for the monochromatic pinhole method. Fankuchen (316) found, however, that spurious halos due to the white radiation maximum could not be eliminated sufficiently by filters in photographing the tobacco mosaic virus. He employed a pentaerythritol crystal condensing monochromator, which produced high intensities and permitted reasonably short exposures, in conjunction with copper radiation.

*Interpretation of the diffraction patterns*

These remarks serve merely as an introduction to the discussion of results which follow. Recent papers by Robertson (430a) and Warren (431) give summaries of methods of interpreting x-ray diffraction patterns. For more extended treatments, one is referred to standard treatises on x-rays (e.g., 307).

*a. Liquids.*—The diffraction patterns produced by liquids are diffuse halos (Fig. 1(a)), typical of

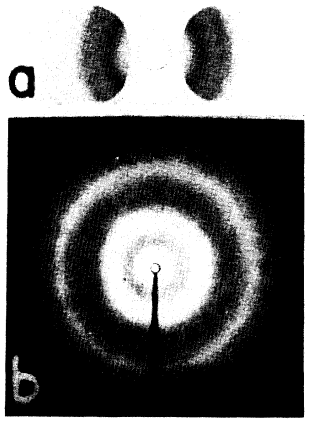



FIG. 1. (a) X-ray diffraction pattern of water, after Warren, reference 431. (b) Powder-type pattern of heat-denatured hemoglobin, after Clark, reference 407.

"amorphous" substances. By the application of Fourier analysis (432), the intensity distribution may be interpreted so as to yield information as to the intermolecular and interatomic distances, at least in the case of sufficiently simple molecules.

*b. Fibers.*—Since the emphasis in the application of x-ray diffraction techniques to biologic problems is at present on the study of long-chain polymers which form fibers, the interpretation of fiber patterns is particularly important.

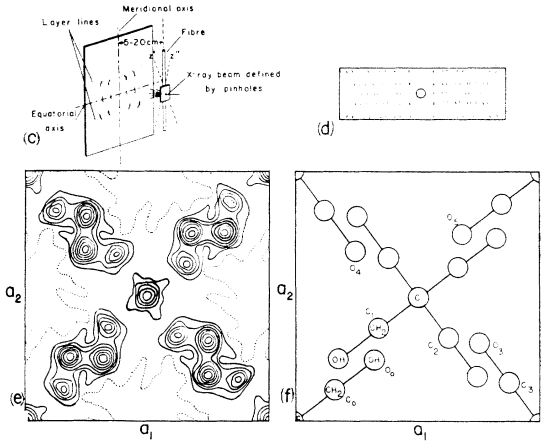



FIG. 1. (c) Schematic representation of fiber pattern technique. (d) Idealized rotation pattern of quartz, after Warren, reference 431. (e) Contour map, and (f) atomic configuration of pentaerythritol, after Llewellyn, *et al.*, reference 436.

Fibers are usually photographed with their axes perpendicular to the x-ray beam and parallel to the plane of the film (Fig. 1(c)). In the accompanying drawings, the fiber axis corresponds to the vertical, or meridional, axis unless otherwise marked. The arrangement resembles that for Laue photographs, except in the use of monochromatic instead of heterochromatic radiation and of a fiber specimen instead of a large, single crystal. Flat films are usually used, but films curved into a cylinder about the specimen as axis may be employed.

The structure of the fiber may range from a single, long crystal to an amorphous array. Neither of these extremes would produce a typical fiber pattern. Since monochromatic radiation is employed, it would be necessary for the single crystal to have a particular orientation with regard to the x-ray beam for constructive interference to occur between reflections from successive planes within the crystal. The probabilities are more against than for such a preferred orientation happening by chance alone. In the case of an ideally amorphous array, there would be no regularly-spaced planes to produce interference maxima—although materials usually classed as amorphous have sufficiently regular inter- or intramolecular spacings to cause them to yield diffraction halos (see Fig. 1(a), pattern for water).

If, instead of being a single crystal or an amorphous array, the fiber is made up of a multitude of crystallites, or of groups of regular planar spacings between molecular or submolecular units, and if these crystallites or multiplanar units are orientated at random, the conditions for constructive interference will be satisfied by some of them, and the familiar Hull-Debye-Scherrer type of pattern will be obtained. Random orientation of crystallites or planar groups will not, however, generally occur about all possible axes within the fiber. Suppose, for example, that there were random orientation only about the fiber axis (meridional axis). The pattern would then be equivalent to a rotation photograph for a single crystal made by rotating it about one of the crystal axes. It would consist of spots along the equatorial axis and at both sides of it on "layer-lines" (which are segments of hyperbolae if the photograph is made on a flat film and straight lines parallel to the equator if it is made on a circular film), with absence of spots on the meridional axis. If, however, random orientation or rotation were to occur only about the equatorial axis, spots would be absent on this axis but would appear on the meridional axis.

In general, there are, of course, an infinite number of possible axes parallel to the plane of the photograph about which the orientation might theoretically be a random one. Should disorientation be confined to rotations about axes in the range $Z''$ to $Z'$ (Fig. 1(c)) the spots corresponding to a $Z$ axis rotation pattern would lengthen into arcs extending from $Z''$ axis to $Z'$ axis rotation spots. As the range from $Z''$ to $Z'$ became greater, the limit would ultimately be approached in which the arcs would fuse into the Hull-Debye-Scherrer rings of completely disorientated crystallites. Summarizing: (a), the appearance of spots or rings in the pattern is indicative of a regularity of structure in the form of groups of equi-spaced planes, or of crystallites, which have sufficiently random orientation to permit reflection of in-phase radiation from some of them; (b), in the usual fiber pattern, the absence or near absence of spots along a particular axis, together with the presence of spots along a perpendicular axis, is indicative of random orientation (i.e., rotation about the first axis; and (c), spots indicate preferred orientation, arcs indicate less regular orientation, and circles indicate completely random orientation.

The positions of the spots, together with the specimen-to-film distance and the wave-length of monochromatic radiation employed provide the necessary data for calculation of the interplanar spacings in the specimen. Distances along the meridian correspond to regularities along the length of the fiber, those along the equatorial axis to spacings across the section of the fiber, and those in intermediate directions to spacings in intermediate directions (provided that the photograph is made with the fiber parallel to the meridional axis). From such spacings, the dimensions of the unit cell may be calculated. From knowledge of the molecular weight and density of the substance under investigation, the number of submolecular or molecular units in each unit cell may be deduced. The fibers usually examined are built up of long-chain polymers, in which the unit cell includes one or more of the polymer residues. The polymeric chains in stretched fibers are usually arranged parallel to the fiber axis or in a spiral configuration along the direction of the fiber axis.

For information as to the complete atomic configuration within the unit cell, it is necessary to have data as to the relative intensities of the spots. For example, see the discussion of rotation patterns from single crystals below.

*c. Small crystals.*—Crystals too small for analysis by rotation patterns may be examined by the Hull-Debye-Scherrer powder method, yielding series of concentric circles on flat films, or approximately straight lines on curved films. The positions of these circles or lines may permit the calculation of unit cell dimensions. The broadness of the lines is an indication of the size of the crystalline particles, both in powder patterns and in the fiber patterns described above. This is true because the greater the number of parallel planes in each crystallite contributing to the interference maxima, the sharper will be the diffraction pattern, an effect analogous to the increase in resolving power of a grating with increasing number of lines. Particle size may be unimportant if it is a result of the method of preparation of the specimen (e.g., grinding), but when it is determined by the natural state of the material (as in colloids or in polymeric chains

photographed by the fiber method), its calculation may be of considerable interest.

*d. Comparatively large crystals.*—If crystals of the order of 0.1 mm in size are available, complete analyses are possible. The usual method is to rotate, or oscillate, the crystal and so to obtain typical "rotation" patterns. Monochromatic radiation, collimated ordinarily by pinholes, is employed. The pattern consists of a series of spots along the layer lines. If the crystal is rotated around three of its axes, the positions of the spots yield complete information as to the size and shape of the unit cell. From the intensities of the spots and the application of Fourier analysis (as it occurs in the theory of scattering of x-rays by electrons within the atom), especially by the methods developed by Patterson (433, 434) and Harker (435), it is possible to deduce electron-density contour maps for the molecule which indicate the positions of all of the atoms, with the exception of hydrogen, within the unit cell. Such contour map for the alcohol pentaerythritol and the atomic configuration deduced from it, as taken from a paper by Llewellyn *et al.*, (436), are shown in Fig. 1(e), (f).

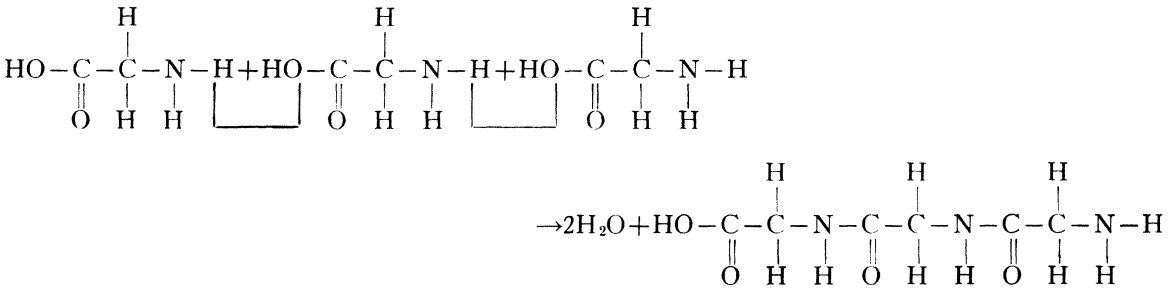
### 2. Results of x-ray investigations

This discussion will be limited to the results of studies of particular biologic interest, and data regarding organic compounds in general will not be considered. For a discussion of the latter field, one is referred to Clark's book (307) and to a review by Mark and Schossberger (317).

*Proteins*

Probably no other class of chemical compounds is of greater importance in biology than the proteins, for they are the substances from which the vast majority of animal body structures are built. The muscles, the connective tissues which hold the organism together, and the epithelial cells which cover its external and internal surfaces are all constituted largely of proteins. As Mathews has aptly put it, even "the blush of a maiden's cheek is due to a red protein circulating in her blood."

A protein may be defined chemically as an organic compound which yields many amino acids on hydrolysis, and the chemical picture of a protein is that of a long chain molecule comprising amino acids linked together through their amino and carboxyl groups by "peptide" linkages. The formation of peptide linkages between amino acids by loss of water is illustrated by the hypothetical example below of the condensation of three molecules of glycine (the simplest of the amino acids) to form a tripeptide. The amino acids in proteins all have the amino group attached to the $\alpha$-carbon atom, and all except the simplest one, glycine, are *l*-amino acids (by which is meant that they have the same spatial configuration about the asymmetric carbon atom as *l*-lactic acid; *not* that they rotate polarized light counter-clockwise). The twenty-two to thirty amino acids of which proteins are built permit almost endless permutations and combinations, providing the chemical basis for the specificity of properties of the proteins from each organism (in fact, from each part of each type of cell in the organism). X-ray analysis as applied to protein structure has been of use principally in establishing the geometrical configuration of the peptide chains under various conditions, in indicating the possibility of condensation into ring structures, and in yielding data as to the sizes of the amino acid residues and of the side chain spacings between protein molecules.



*a. Molecular configuration of silk fibroin.*—The diffraction pattern of silk fibroin (Fig. 2) is a complex array indicative of a considerable degree of molecular orientation, with a regularity of spacing of roughly 7A (two residues) along the fiber axis and of 10A and 4.5A perpendicular to it (318–320). Meyer and Mark (321) concluded from such patterns that the protein molecules in silk fibers are long chains parallel to the fiber axis, each fiber being *a kind of thread spun from molecules*. This view was consistent with the conventional chemical notion of protein molecules as long polypeptide chains, and has since been developed most intensively by Astbury and coworkers (423). The interpretation of the x-ray data in terms of molecular configuration is indicated in Fig. 2.

*b. Keratin patterns and Astbury's molecular folding hypothesis.*—When fibers composed largely of keratin (e.g., wool) were examined, they were found to have diffraction patterns indicative of irregular molecular orientation distinctly different from that in silk fibers (Fig. 2(d)); but when the keratin fibers were *stretched* (Fig. 2(b)), they produced patterns *somewhat similar to those of silk* (322–328, 426). On release of tension, permitting the keratin fibers to contract, the patterns returned to their original, less regular type. Hence the change brought about by stretching was evidently reversible. As a result of these experiments, and of numerous other investigations of the patterns produced by keratin (324, 327–333), myosin and muscle (334–341), collagen (333, 342–347), and denatured globular proteins (342, 343, 348), Astbury (342) developed the theory that the fibrous and denatured globular proteins (as distinguished from nonfibrous, or globular, or corpuscular proteins) can exist in four principal states of folding, represented schematically, together with the corresponding x-ray patterns, in Fig. 2. According to this point of view, keratin molecules in the normal, unstretched state are in the partially folded ("$\alpha$") form, and when keratin is stretched (e.g., under water), the molecules unfold and become fully extended ("$\beta$" form). The unstretched ("$\alpha$") form is capable of being shrunk to considerably less than its usual length, the molecules then being presumed to be further folded, or "supercontracted."

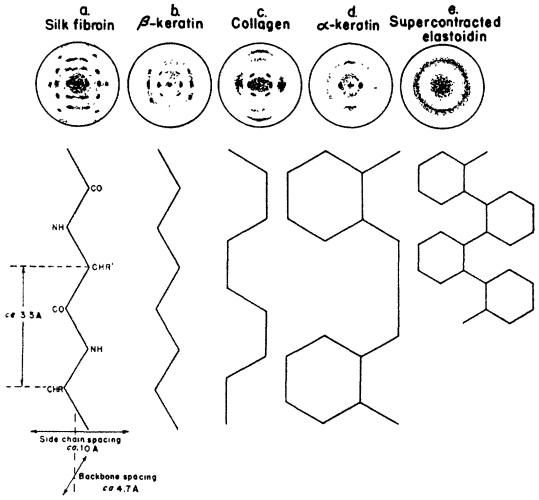



FIG. 2. Idealized x-ray fiber patterns of various protein fibers and their interpretation in terms of the configuration of the protein molecules.

*The extreme extensibility of such materials as hair is thus explained in terms of the spring-like folding and unfolding of the molecules of which they are composed.* The contraction of muscle tissue is similarly accounted for by folding of its myosin molecules. In stretched muscle, the molecules are evidently in the partially folded ("$\alpha$" form), while in contracted muscle they are "supercontracted."

The long polypeptide chains, in the "$\beta$" configuration, lying side by side, are presumed to be held together by interaction between their side chains (*R* groups), forming grid-like sheets which fold as units on contraction of the molecules to the "$\alpha$" form (compare the "cyclol" hypothesis, discussed below).

It is conceivable that the diffraction patterns observed with hair, etc., might be due to histological components other than molecules of the elastic protein material itself. In order to investigate this possibility Woods (351, 352) devised methods of examining the isolated individual cells of hair, wool, etc. by orientating them in an electric field and drying them while they were so orientated. The results confirmed those obtained with intact tissues. Hence the diffraction patterns evidently cannot be attributed to intercellular material.

*c. Proteins of the collagen type.*—Collagen and related proteins (from connective tissue, tendon, cartilage, etc.) comprise a separate class in which

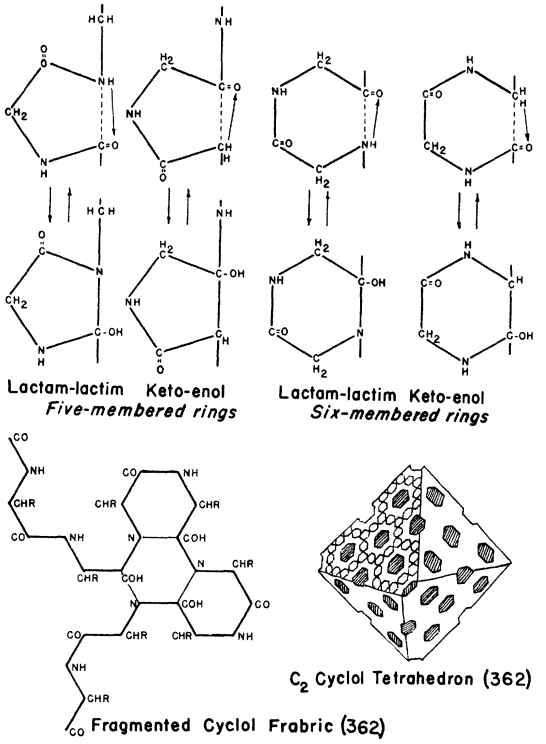



FIG. 3. Formation of folded structures by polypeptide chains.

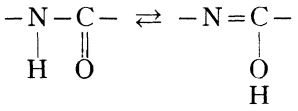
the extended form appears to be intermediate between the "$\alpha$" and "$\beta$" forms of keratin (333, 342–347), as shown in Fig. 2. They undergo contraction under appropriate conditions, but apparently do not stretch beyond the partially-folded form. Their diffraction patterns indicate an average residue length in the direction of the fiber axis of about 2.9A, with a side-chain spacing of about 10.4A and a backbone spacing of about 4.4A. Astbury *et al.* explain the residue spacing and the inability to stretch into a fully-extended "$\beta$" chain by a partial *cis* configuration instead of the *trans* configuration of $\alpha$-keratin. For details, see a recent paper by Astbury and Bell (526).

The stereochemistry of the polypeptide chain folding theory is discussed in papers by Neurath *et al.* (350).

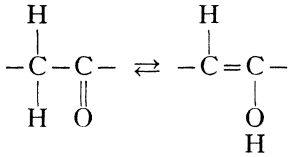
*d. Effect of steam in permanently setting keratin molecules and in causing their supercontraction.*—If stretched hair is steamed, it stays permanently elongated ("$\beta$" state), evidently because of hydrolysis of the cross linkages that ordinarily cause the contraction and folding which bring about return to the "$\alpha$" state (349). Thus, x-ray diffraction patterns indicate that a woman's "permanent wave" is explained by the setting of the unfolded keratin molecules in the extended "$\beta$" state when her hair is steamed while stretched on an appropriate form. Stretched hair which has not been steamed sufficiently in the stretched state to give it a permanent "set" will contract to either the "$\alpha$" or "supercontracted" state on being steamed after release of tension.

*e. Patterns of denatured globular proteins.*—In contrast with the fibrous proteins, those of the "globular" type give no x-ray evidence of extended chain configuration in their normal states, If, however, globular proteins are "denatured," they yield diffraction patterns indicative of the presence of extended polypeptide chains in the "$\beta$" configuration (343). Furthermore, it has been found possible to spin urea solutions of the seed globulins into artificial elastic fibers which can be stretched to yield fiber patterns of the "$\beta$" type (348). These results may be interpreted as indicating that the polypeptide chains actually exist in natural globular proteins, but in a three-dimensional folded system as contrasted with the two-dimensional folded system postulated for fibrous proteins in the "$\alpha$" configuration, or they may be explained on the basis of the "cyclol" hypothesis, discussed below.

*f. Mechanism of folding; the "cyclol" hypothesis.*—Astbury and co-workers deduced that the folds in "$\alpha$" keratin, etc., might be in the form of hexagonal rings (325, 327, 334), and tautomeric changes of the lactam-lactim type



were suggested as the possible mechanism of ring closure (353–355). Hydrogen bonds (356–358, 441) and keto-enol (359) tautomerism



have also been postulated as responsible for ring formation. Four possible rearrangements of the lactam-lactim and keto-enol types, with resulting condensation of polypeptide chains into five- and six-membered rings, are illustrated in Fig. 3.

Wrinch adopted the lactam-lactim proposal in her "cyclol theory" of protein structure, in which the molecules are presumed to form continuous fabrics (Fig. 3) of hexagonal rings (360–366) as contrasted with the grid-like structure postulated by Astbury for "$\beta$"-keratin. In globular proteins, for example, the fabric is thought of as constituting the sides of hollow, cage-like tetrahedra, capable of packing through association of their faces or edges (367). Geometrical considerations require that the number of amino acid residues present in each tetrahedron be $72n^2$. The various possible cyclol tetrahedra are designated by $C_1$, $C_2$, etc., the subscripts corresponding to "$n$." In the case of $C_2$, the number of residues is $72\times 2^2=288$, which gives a molecular weight of 36,000 if the mean residue molecular weight is taken as 125. This is strikingly close to the value 35,200 found by Svedberg and others, by ultracentrifuge methods,* to be the fundamental unit of molecular weight among the proteins. Furthermore, certain proteins, such as myoglobin and *Lampetra* erythrocrurin, are known to have molecular weights of half this value, and these can be accounted for by combinations of two $C_1$ units. Thus, on the basis of the cyclol theory, the fundamental structural limit to the size of the protein molecule becomes a particular number of amino acids ($C_1=72$, $C_2=288$, etc.), rather than a particular molecular weight; altogether a more reasonable assumption, since the theory demands such a limit on geometrical grounds, whereas it is difficult to conceive a mechanism which would impose corresponding limitations directly on the molecular weight. Lastly, the deductions of Bergmann and Niemann (437–439), from data as to products of hydrolysis of proteins, molecular weights, etc., that the total number of amino acids in any protein may be represented by $2^n3^m$ (where "$n$" and "$m$" are positive whole numbers), and the total number of residues of a particular amino acid by $2^{n'}3^{m'}$ (where "$n'$" and "$m'$" are either zero or positive whole numbers) fits in quite well with the cyclol theory, since the values for total numbers of amino acids calculated for actual proteins on this basis turn out to be 288, or whole number multiples thereof.

In a fabric such as that postulated, all of the $R$ groups of the amino acids, which give to the protein its specific properties, lie on one side of the fabric plane. This implies important surface characteristics, and provides a possible explanation of protein synthesis *in vivo* by a process somewhat analogous to that of crystal growth (368). The hollow structure of the tetrahedra, spotted with openings required by the geometry of packing of the hexagonal rings, would afford opportunity for the passage of water or other molecules into the cages, and would permit the existence of a "foreign population" of molecules within the actual protein structure (362). Denaturation is explained on the cyclol hypothesis by fragmentation of the cyclol fabric, with release of polypeptide chains.

Altogether, the theory seemed to account for many properties of proteins, and numerous arguments were advanced in its favor in the papers cited and in further publications by Wrinch (369), Langmuir (370), Talmud (371), etc. Nevertheless, severe objections to the theory were soon forthcoming. Pauling and Niemann (372) have summarized these objections (see also discussion of insulin, below). Briefly, their main points of criticism are as follows: (1) the heat of denaturation of proteins is too small to correspond to the energy difference between cyclols and unfolded polypeptide chains, like $\beta$-keratin; (2) thermochemical data indicate cyclols to be less stable than polypeptide chains; (3) impossible stereochemical configurations are required by the cyclol theory; and (4) chemical properties implied (due to the large number of OH groups) are not found experimentally. One judges that Wrinch herself is dissatisfied with the theory from the fact that she has recently postulated an entirely different fabric involving keto-enol instead of lactam-lactim tautomerism (369a). Probably the most straightforward objections to the cyclol hypothesis, and indeed to the type of folding postulated for $\alpha$-keratin, are those based on stereochemical packing difficulties, discussed

* To be discussed later.

in papers by Neurath *et al.* (350) previously referred to. The whole question is still very much unsettled. For an interesting discussion of the present status of the problem, one is referred to a review by Astbury (527) in a symposium on the hydrogen bond.

*g. Textile fibers.*—Investigations of keratin, wool, etc., are of great interest in connection with textile problems (349, 380–382). Astbury's data (349) show that deamination of wool fibers prevents the molecules from being "set" by steam in the "$\beta$" configuration. His data also indicate that the penetration and interaction of acid dyes with basic groups along the polypeptide chains within the fiber structure are limited by the size and mobility of the dye molecules. X-ray diffraction studies have been found useful in the development of synthetic polymers, such as "nylon" and "rayon," in affording a means of checking the fiber structures and comparing them with those of the naturally occurring substances for which substitutes are desired (307).

*h. Patterns of built-up protein films.*—Astbury *et al.* (373) have examined the x-ray diffraction patterns and optical properties of built-up films comprising 600 to 1764 monomolecular layers of egg albumin (a globular protein) formed by passing a metal slide repeatedly through a monomolecular layer of the protein (method of Blodgett (374)). The films were stripped, prior to examination, from the metal bases on which they were formed. The data indicated that the monomolecular layers comprised polypeptide chains lying approximately parallel to the direction of motion of the slide through the film. The thickness of each layer, corresponding to the side-chain spacing, was about 9.5A, and this value was confirmed by optical and mechanical measurements. Stenhagen (375) also found a 9.5A layer thickness for 40-layer films of lipoprotein examined directly on the metal slide. These investigations indicate the existence of folded polypeptide chains in globular proteins. On the other hand, Clark and Ross (376) did not find patterns indicative of the 10A side chain spacing characteristic of polypeptide chains in examination of built-up films of egg albumin comprising 30–70 layers, but only a spacing of 73.3A, which they believed might be consistent with a cyclol structure.

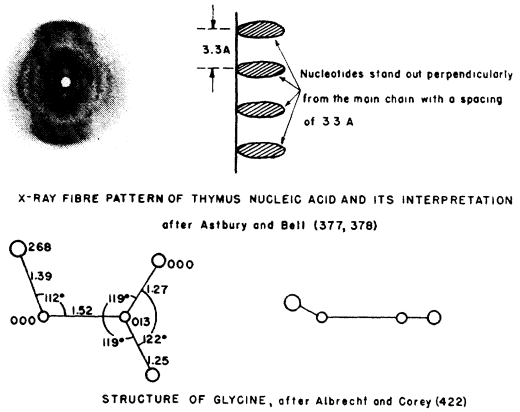



FIG. 4. X-ray diffraction studies of thymus nucleic acid and glycine.

*i. Nucleoproteins.*—The nucleus of the cell, which is the part primarily concerned with cell division and with control of cellular metabolism, is composed largely of proteins combined with nucleic acids. The latter are complex structures built of nitrogenous ring compounds in combination with sugars and phosphoric acid. The chromosomes in the nucleus—the rod-shaped bodies in which apparently lie the secrets of the transmission of hereditary characteristics—are almost entirely nucleoprotein. It would seem, therefore, that an understanding of the structure of these molecules would be of great help in elucidating the mysteries of reproduction, heredity, growth and differentiation.

A start has been made in the study of such compounds by Astbury and co-workers (377, 378) and by Mazza and Tappi (379). Astbury and Bell found, for thymonucleic acid,* a spacing along the molecule axis of 3.3A, and side dimensions of about 16A and 8A. These spacings led them to deduce a structure for thymonucleic acid of the type shown in Fig. 4. As they pointed out, the extremely interesting feature of this hypothecated structure is that the separations of the nucleotides from each other (3.3A) correspond almost exactly to the distances between successive amino acid residues in the extended polypeptide chain (3.5A, see Fig. 2). Hence, one may deduce that this spatial design permits ready interaction between the nucleic acids in the

* Employed as sodium thymonucleate.

chromosomes and the side chains of neighboring protein molecules, as a basis for the beginning of the growth process. Astbury *et al.* succeeded in studying compounds formed by the interaction of thymus nucleic acid with the proteins clupein and edestin. Clupein thymonucleate showed a pattern in all respects similar to that of thymus nucleic acid; that of edestin thymonucleate was, however, different and somewhat difficult of interpretation.

In view of the part thought to be played by lactam-lactim and keto-enol tautomerism in the formation by proteins of polypeptide rings and of the cyclol protein fabric, it would be interesting to consider similar possibilities in the interaction of nucleic-acid constituents with proteins and with each other. The purines and pyrimidines of nucleic acids have extraordinarily numerous possibilities of tautomeric rearrangements (see, for example, the discussion of ultraviolet absorption spectra, below).

*j. Insulin.*—Several of the globular proteins are obtainable as crystals sufficiently large for rotation analyses. Of these, insulin has been studied in the greatest detail (383–388). The sharpest, most satisfactory patterns of these globular protein crystals are obtained if they are photographed while kept moist (e.g., with some of the mother liquor from which they were crystallized). Crowfoot and Riley (388) found the cell dimensions of insulin to be: $a=83.0$A, $c=34.0$A, molecular weight $=52{,}400$, for wet crystals; and $a=74.8$A, $c=30.9$A, molecular weight $=37{,}600$, for dry crystals; space group $R3$. The configuration of insulin has been worked out by Patterson-Harker analysis, but a stormy controversy has arisen over the interpretation of the results. Wrinch and Langmuir (389–396) claim that they strongly support a cyclol structure. Their conclusions have been severely criticized by Bernal, Riley and Fankuchen (397–400), Bragg (401), Robertson (402), and Pauling and Niemann (372).

*k. Pepsin.*—Rotation patterns of wet crystals of pepsin lead, according to Bernal and Crowfoot (383), to unit cell dimensions of: $a=116$A, $b=67$A, $c=460.8$A. Wrinch (403) interprets the data as indicative of a cyclol $C_2$ structure, with 288 amino acid residues in the pepsin molecule.

*l. Hemoglobin.*—This protein has been investigated by George (404), Wyckoff and Corey (405), Bernal *et al.* (406), and Clark and Shenk (407). The last authors showed that denatured hemoglobin gives a pattern very similar to that of denatured egg albumin, indicative of an unfolding of the polypeptide chains as observed for other denatured globular proteins (343). They found marked differences in the patterns of hemoglobins from different animals (rats, horses, pigs). The results of rotation analyses (406) are shown in Table III.

*m. Other globular proteins.*—Results of rotation analyses of chymotrypsin (406), lactoglobulin (409) and excelsin (348) are summarized in Table III. Clark and Shenk (408) found that the sharpness of the rings obtained with denatured egg albumin varied with the denaturing agent used. The same authors (410) investigated the mechanism of the hardening action of formaldehyde on globular and fibrous proteins. They concluded that formaldehyde acts on the amide nitrogens to tie adjacent chains together.

Powder-crystal analyses have been obtained of tobacco seed and squash seed globulins (343), chymotrypsinogen (333), and the Bence-Jones protein (411), for the results of which see Astbury and Bell (506).

*n. Virus proteins.*—X-ray studies of the tobacco mosaic and cucumber virus proteins (412–415) show that their long molecules are packed closely in hexagonal symmetry in planes perpendicular to their length, with no apparent regular arrangement along their lengths. The molecules appear to be rod-shaped, with widths of about 150A and lengths at least 1000A. On the other hand, examination of a tomato virus protein by Bernal *et al.* (528) showed its molecules to be globular, with diameters of about 340A.

Particular interest has centered in the virus proteins because at one time their ability to reproduce under certain conditions led to their being regarded as living organisms, whereas it is now known that they are simply giant protein molecules.

*o. Amino acids.*—While the fiber, powder and rotation patterns described above have yielded interesting and useful information about the general structure of the protein molecule, it seems

TABLE III. *Summary of x-ray data regarding proteins. All dimensions are in* A. *Z = number of molecules per unit cell.*

| SUBSTANCE | X-RAY METHOD | SPACINGS | | | UNIT CELL | | | | CRYSTAL TYPE OR SPACE GROUP | Z | REFERENCES |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | BACK-BONE | SIDE CHAIN | RESIDUES (ALONG MAIN CHAIN) | *a* | *b* | *c* | *β* | | | |
| Silk fibroin (Bombyx mori) | Fiber | 4.27 to 5.29 | 9.17 to 9.45 | 6.7 to 7.25 (2 residues) | | | | | | | 318–321 |
| Silk fibroin (Satonia) | Fiber | 4.50 | | 6.95 to 7.45 (2 residues) | 6.46 | 7.2 | 15.34 | 61°10′ | Monoclinic | 8 | 319, 320 |
| Feather keratin (β-type) | Fiber | 4.5 | 11.3 (many other spacings) | 3.08 | | | | | | | 329, 333, 423 |
| α-keratin (α-myosin)* | Fiber | | 9.8 | 5.06 (3 residues) | 27 | 10.3 | 9.8 | | Orthorhombic | | 324, 327 |
| β-keratin (β-myosin)* | Fiber | 4.65 | 9.7 9.8 | 3.33 | 9.3 | 6.66 | 9.7 | | Orthorhombic | | 324, 327, 328 |
| Collagen | Fiber | 4.4 | 10.4 | 2.9 | | | | | | | 526 |
| Supercontracted proteins | Fiber | Disoriented pattern with some indications of β-type structure | | | | | | | | | 324, 327, 334 |
| Denatured proteins | Fiber | Powder type patterns 4.5 | 10 | 3.6 | | | | | | | 342, 343, 348 |
| Fibrin (fibers) | Fiber | 4.7 | 10 | 3.35 | | | | | | | 546 |
| Built-up egg albumin | Fiber | | 9.5 | | | | | | | | 373 |
| Thymus nucleic acid (stretched film) | Fiber | Side spacing =16.2 | | Spacing along mol. axis =3.3 | | | | | | | 377, 378 |
| Insulin (wet) (dry) | Rot. Crys. | | | | 83.0 74.8 | | 34.0 30.9 | | *R*3 | 1 | 388 |
| Pepsin (wet) | Rot. Crys. | | | | 116 | 67 | 460.8 | | | | 383 |
| Hemoglobin (wet) (dry) | Rot. Crys. | | | | 109 102 | 63.2 56 | 54.2 49 | 112° 134° | *C*2 *C*2 | 4 4 | 406 |
| Chymotrypsinogen (wet) (dry) | Rot. Crys. | | | | 49.6 45 | 67.8 62.5 | 66.5 57.5 | 102° 112° | $P2_1$ $P2_1$ | 4 4 | 406 |
| Lactoglobulin Tabular (wet) Tabular (dry) Needles (wet) Needles (dry) | Rot. Crys. | | | | 63.5 59 63.5 54 | 63.5 59 63.5 54 | 145.0 105 125.0 125 | | $P2_12_12_1$ $P4_22_1$ | 8 8 8 8 | 409 |
| Excelsin | Rot. Crys. | | | | 86 | | 208.2 | | *R*3 | 1 | 348 |

* α-myosin and β-myosin are similar to α-keratin and β-keratin. See reference 334.

reasonable that the elucidation of many details of protein structure will have to await complete analyses (e.g., by the Patterson-Harker method) of individual amino acids. This approach has been recommended by Corey (416) in his recent review of x-ray studies of interatomic distances in proteins.

Amino acids have not, thus far, received as great attention as proteins. Bernal published some preliminary data on 15 amino acids (417), and one paper has appeared on creatinine (418). The most thorough studies have been those of glycine (419–422). Albrecht and Corey (421) have worked out the crystal structure of glycine in detail from complete Patterson-Harker analysis of intensity data (see Fig. 4).

*p. General conclusions regarding protein studies.* —The results discussed above are summarized in Table III. In considering this table, it is evident that the conclusions arrived at from examination of diffraction patterns of proteins are frequently based on rather confusing and unsatisfactory data. This is hardly surprising, considering the complexity of protein structure. The remarkable fact is that it has been possible to propose interpretations which seem fairly reasonable.

That the stretching of fibrous proteins is associated with some kind of unfolding of polypeptide chains seems fairly well established. The exact nature of the folding in contracted fibers—whether it be a crumpling of grid-like sheets of long molecules, or a contraction to form five- or six-membered polypeptide rings—is still a matter of debate. It also seems established that globular

proteins yield polypeptide chains on denaturation by certain agents. But, again, the question of the actual structure in the intact protein—cyclol fabric or folded polypeptide chains—is still not satisfactorily answered. With the intensive effort that is being devoted to protein and amino acid studies at the present time, one may expect conclusive evidence to be forthcoming within the not too far distant future.

In addition to the reviews already mentioned (307, 378, 416), summaries dealing particularly with x-ray studies of the proteins have been published by Astbury (423–426, 440, 529), Krüger (427), and Bernal (412–414).

*Carbohydrates*

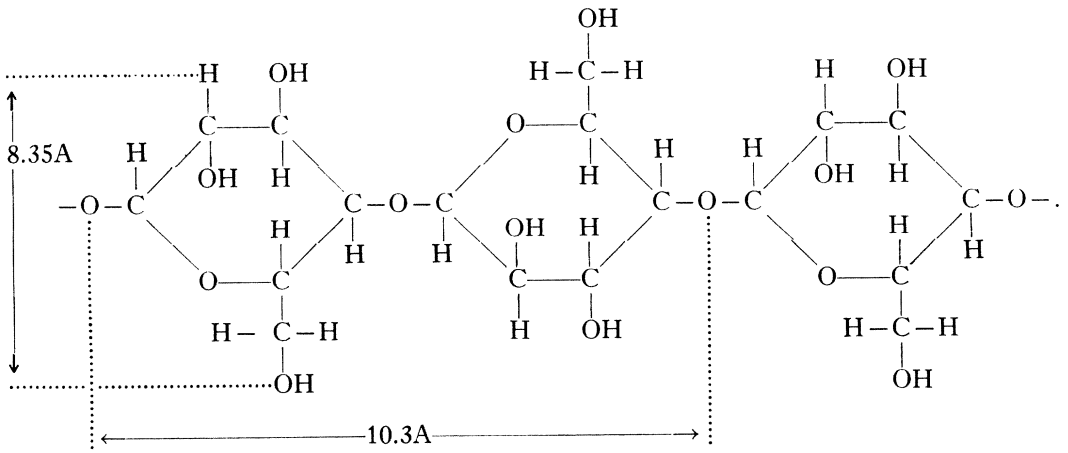
A second great class of compounds of particular biologic interest is the carbohydrates, compounds of carbon, hydrogen and oxygen, which are the chief source of energy, through the usual metabolic processes, in animal organisms, and the chief structural materials in plants. Many of the carbohydrates (e.g., pentose and hexose sugars) are small molecules of comparatively simple structure, but others (such as starch and cellulose) are polymers of which the structure has been comparatively obscure until recently. It is particularly in studies of carbohydrates of the latter type that x-ray methods have been of value.

*a. Cellulose.*—Cellulose is the principal structural material in plants. It has the empirical formula $(C_6H_{10}O_5)_x$, and the structural formula shown below, as deduced from chemical data (the dimensions shown being from x-ray data). It is a polymer of glucose residues.

The x-ray data of Meyer and Misch (442) and others (443, 444) indicate that there are four ($C_6H_{10}O_5$) residues to a monoclinic unit cell, of dimensions $a=8.35$A, $b=10.3$A (fiber axis), $c=7.95$A, $\beta=84°$. Sponsler and Dore (447) and Sauter (448, 449) obtained diffraction patterns indicative of a unit cell of somewhat different dimensions (Sauter: $a=10.48$A, $b=10.4$A, $c=11.8$A, $\beta=85°$, 8 residues per unit cell; Sponsler: $a=10.80$A, $b=10.25$A, $c=12.20$A). Gross and Clark (445, 446) believe that their investigations establish the validity of the unit cell of Meyer *et al.* (see also 450).

Slight lengthening of the lattice on absorption of water of hydration has been described (451). Similar effects have been observed on treatment of cellulose with ammonia (452), the "*b*" axis remaining unchanged and the "*a*" axis lengthening from 8.3 to 9.83A. The dimensions of the unit cell in various cellular derivatives such as cellulose nitrate (453) and cellulose acetate (454) have been measured. Gundermann (455) found a 15A spacing in certain derivatives. For a discussion of such investigations, and in particular of x-ray changes during various chemical reactions of cellulose, one is referred to papers by Hess and Trogus (456–458) and Schramek (459).

With regard to the lengths of the long chain molecules and the method of packing, there is considerable difference of opinion. By estimates of particle size from diffraction band widths, Mark *et al.* (460, 461) concluded that cellulose



structure comprises micellar units about 500× 50×50A. On the other hand, viscosity measurements indicate a molecule of size about 4000× 8×8A (462). Furthermore, Farr and Eckerson (463) found, by optical and x-ray examination of plant cell walls, evidence indicating that cellulose in such structures is in the form of parallel crystals each about 15,000A long and 11,000A in thickness. Investigations of such crystals (which are visible with the aid of a microscope) by Clark (462) and others (464) indicated that they are not built up from smaller crystals but are simply aggregates of long chain cellulose molecules laid side by side parallel to the long axis of the crystal. The fact that the crystalline particles were not discovered sooner is attributed to complications introduced by a pectin-like cementing substance which holds them together. Only after treatment of the fibers to remove this substance (e.g., with concentrated HCl) do the crystals appear on microscopic examination. Ground fragments of untreated cotton fibers exhibit the same x-ray powder photographs as the crystals obtained by removal of the pectin-like substance, indicating that the crystals are actually present as such in the fibers before treatment (462).

In this connection, Wergin's (465) x-ray diffraction studies of cottonseed hairs and *Avena* coleoptiles at various stages of their growth are of interest. Prior to about the 35th day, only powder-type patterns, indicating spacings of 3.75 and 4.20A, were obtained with cottonseed hairs. Then the patterns indicative of longitudinally orientated crystals of cellulose began to appear, and the ring patterns began to disappear, until ultimately only the cellulose crystal patterns remained. The results with *Avena* were similar. Wergen believes that his data indicate that a primary crystalline substance, with unorientated crystals, appears first, followed later by orientated cellulose crystals. Studies such as the above, as well as investigations of Sponsler and Dore (466), Sauter's (467) rotating goniometer fiber diagrams of natural and hydrated cellulose, Clark and Parker's (468) examinations of small-angle diffraction patterns of celluloses and rayons, etc. indicate a particle structure in which the particles are composed of very long molecules laid side by side, rather than the micellar structure of Meyer and Mark.

The most reasonable explanation of cellulose structure seems to be the "network" theory, developed by Kratky and others (469–477), according to which the long molecules of cellulose form a net-like structure, in some portions lying side by side in parallel bundles which coalesce to form crystalline particles or micelles. Overlapping chains help to bind the crystallites or micelles together, and amorphous material dispersed about the crystals, etc. acts as a further binding medium. Kratky *et al.* (478, 479) have used finely divided gold distributed in fibers of ramie to enhance the diffraction pattern; and by this means, and with measurements at small diffraction angles, have obtained values of the micellar sizes, or crystallized areas. These and data from numerous other sources indicate, according to Clark (307), that (a) the long chains of cellulose molecules have degrees of polymerization ranging from 150 (corresponding to 750A) to 2000 (corresponding to 10,000A), (b) that short chains having 700–800 residues, and resulting poor technical properties, occur when cellulose undergoes many kinds of technical treatment, and that (c) when the chain length approximates the micelle length (600–1000A) the tensile strength of the fibers is less, because there is less overlapping of chains from micelle to micelle.

Among other papers on x-ray studies of cellulose which should be mentioned are that of Sisson and Saner (480) on cellulose swelling under the action of quaternary ammonium hydroxides, those of Sisson (481, 538) on the orientation of crystallites in cellulose fibers, in which he found random orientation about the "*b*" axis; that of Meyer *et al.* (482) on regenerated cellulose, that of Clark and Parker (483) on the action of liquid ammonia on cellulose and cellulose derivatives, and several papers on the x-ray examination of de-lignified cellulose (539–541).

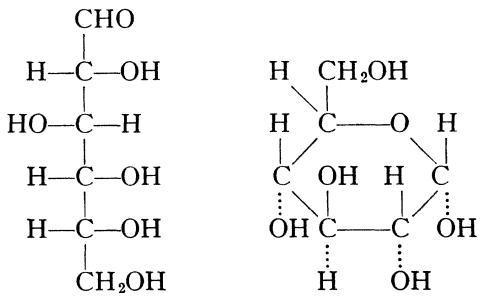
The whole cellulose structure question is so important from the technical and industrial standpoint that many reviews have been published. In addition to those already mentioned, attention should be directed to Sisson's x-ray studies of cellulose derivatives (484) and his discussion of the various theories of cellulose struc-

ture (485), to papers by Preston (486), Kratky *et al.* (487, 488), Staudinger (489), Meyer (490, 491), and to a recent brief review by Wanda Farr (492).

*b. Chitin.*—Chitin is similar to cellulose in chemical structure, except that it contains nitrogen. It is a horny, colorless substance that forms the principal part of the outer coat of insects, crustaceans, etc. It is also found to a limited extent in plants. Its crystal structure has been investigated by several workers (493–498), and it appears that the unit cell is orthorhombic, with dimensions as follows: $a=9.25$ to 9.7A, $b$ (fiber axis) $=10.4$ to 10.46A, $c=10.25$A (494–496). The long chain molecule, similar to cellulose, is apparently composed of from 100 to several hundred glucose-amine groups (498). Nitrochitin gives the same pattern as chitin according to Meyer *et al.* (498) but slightly different spacing according to Clark and Smith (495). The identical x-ray patterns of natural chitin and chitin treated with NaOH (498) indicate that it does not undergo mercerization as does cellulose. Chitin from plant sources gives the same patterns as that from animal sources (496, 497). The structure of chitosan, a hydrolysis product, has also been investigated (495) and found to be a monoclinic unit cell with dimensions $a=8.9$A, $b=10.25$A, $c=17.0$A, $\beta=88°$, with a 75A lateral spacing between the crystallites.

*c. Other polysaccharides.*—The three most important polysaccharides in foodstuffs are cellulose, starch, and glycogen. Of these, cellulose has been discussed above. Starch yields one of several different powder diffraction patterns (three, or possibly four) depending upon its source and previous treatment (499–504). It does not produce fiber patterns, like cellulose and chitin, despite the fact that (like cellulose) it is a polymerization product of glucose residues. No very informative interpretation of the data for starch has as yet been forthcoming. Centola reports that nitrated starch has a pattern similar to that of nitrocellulose (505). Glycogen appears to yield only amorphous patterns (499). Data for several other polysaccharides will be found in a recent review by Astbury and Bell (506). In general, the patterns obtained are not easy to interpret. May and Graf (506a) state that animal polysaccharides yield only amorphous photographs while plant polysaccharides give crystal patterns; an interesting generalization if it can be substantiated.

*d. Monosaccharides.*—These are the simple, nonhydrolyzable sugars. Of them, three hexoses, as follows, dextrose, galactose and levulose, are present in large quantity in the animal organism and the foodstuffs it ingests. It is mainly through the metabolism of dextrose (or *d*-glucose) by its cells that the animal organism receives its energy. The structural characteristics of these monosaccharides are consequently of great interest. Until quite recently, it was believed that they were open chains, but chemical evidence has led, in the last few years, to the acceptance of a ring structure (pyran ring) as shown below. Here x-ray evidence has been of assistance in helping to establish the validity of the pyranose (pyran ring) formula.




Old, Open-chain Formula  Pyranose Formula

Structure of $\alpha$-*d*-Glucose

The general argument from the x-ray data centers about the measurements of unit cell dimensions, and attempts to fit various postulated types of molecules into such cells. The structural problem does not end with the acceptance of a pyranose formula, however. The question immediately arises as to whether the ring is flat, or puckered (the carbon bonds being disposed in the latter case at the tetrahedral angle of 109°). If the puckered, or strainless Beyer type of ring structure, is accepted, there arise further stereochemical questions as to the disposition of the various ring atoms with respect to each other (thus, there are two *trans* forms, six *cis* forms, and two forms with the carbon atoms in a plane and the oxygen outside—in all, ten steric forms of the Beyer type structure—

TABLE IV. *Summary of x-ray data for carbohydrates. All dimensions are in* A. *Z = number of molecules per unit cell.*

| SUBSTANCE | DENSITY | DIMENSIONS OF UNIT CELL* | | | | CRYSTAL SYSTEM | SPACE GROUP | Z | REFERENCES |
|---|---|---|---|---|---|---|---|---|---|
| | | *a* | *b* | *c* | *β* | | | | |
| Cellulose | 1.52 | 8.3 | 10.3 (fiber axis) | 7.9 | 84° | Monoclinic | $P2_1$ | 4 | 518 |
| | | 10.48 | 10.4 | 11.8 | 85° | | | 8 | 448, 449 |
| | | 10.80 | 10.25 | 12.20 | | | | | 477 |
| Hydr. cellulose | 1.56 | 8.14 | 10.3 | 9.14 | 62° | Monoclinic | | | 519, 520 |
| Chitin | | 9.25–9.7 | 10.4–10.46 (fiber axis) | 19.25 | | Orthorhombic | | | 493–498 |
| Chitosan | | 8.9 | 10.25 | 17.0 | 88° | Monoclinic | | | 495 |
| Cellobiose | 1.56 | 5.0 | 13.2 | 11.1 | 90° | Monoclinic | | | 517 |
| *α-d* Glucose | | 10.45 | 14.85 | 2.48 | | Orthorhombic | $P2_12_12_1$ | 4 | 521 |
| | 1.54 | 10.40 | 14.89 | 4.99 | | | | | 517 |
| *α-d* Glucose monohydr. | 1.57 | 8.72 | 5.03 | 9.59 | 97°59′ | Monoclinic | $P2_1$ | 2 | 521 |
| Sucrose | 1.59 | 11.0 | 8.7 | 7.65 | 103.5 | Monoclinic | | | 516 |
| Mannose | 1.5 | 7.62 | 18.18 | 5.67 | | Orthorhombic | | | 516 |
| Sorbose | 1.65 | 6.12 | 18.24 | 6.43 | | Orthorhombic | | | 516 |
| *d*-Sorbose | 1.63 | 18.01 | 6.51 | 6.26 | | Orthorhombic | $P2_12_12_1$ | 4 | 511 |
| Fructose | 1.598 | 8.06 | 10.06 | 9.12 | | Orthorhombic | | | 517 |
| *β-l* Arabinose | 1.63–1.67 | 6.51 | 19.43 | 4.85 | | Orthorhombic | $P2_12_12_1$ | 4 | 507 |
| *α-d*-Xylose | 1.53 | 12.64 | 9.20 | 5.62 | | Orthorhombic | $P2_12_12_1$ | 4 | 507 |
| *d*-Rhamnose hydr. | 1.44–1.47 | 7.96 | 7.95 | 6.71 | 95°25′ | Monoclinic | $P2_1$ | 2 | 507 |
| *α-d*-Galactose | 1.58 | 12.68 | 7.78 | 7.71 | | Orthorhombic | $P2_12_12_1$ | 4 | 511 |
| *α-l*-Fucose | 1.49–1.52 | 14.3 | 7.6 | 6.6 | | Orthorhombic | $P2_12_12$ | 4 | 511 |
| *l*-Ascorbic acid | 1.74 | 16.95 | 16.32 | 6.38 | 102.5° | Monoclinic | $P2_1$ | 4 | 512 |

* Unit cell dimensions, etc., for the following derivatives have also been published: *d*-galacto ascorbic acid monohydrate, *d*-imino-galacto ascorbic acid, dimethyl-*d*-gluco ascorbic acid, (508, 512); *β*-methyl-*l*-arabinoside, *α*-methyl-*l* fucoside, *α*-methyl-*d*-galactoside monohydrate, *α*-methyl-galactoside-6-bromohydrin (511); *α*-methyl xyloside (510); *β*-methyl xyloside (509); *α*-methyl mannoside, *α*-methyl-manno pyranoside, *α*-methyl-manno furanoside (514); 2 : 3 : 4-trimethyl-*α* lyxose, 2 : 3 : 6-trimethyl-*α* glucose; 2 : 3 : 4 : 6-tetramethyl-*α* glucose; 2 : 3 : 4 : 6-tetramethyl-*α* galactose, 2 : 3 : 4 : 6 : 7-pentamethyl-*β*-*d*-gluco heptose, 2 : 3 : 4-trimethyl-*β*-methyl glucoside, 2 : 3 : 4 : 6-tetra-*α*-methyl mannoside, and 1 : 3 : 4-trimethyl fructose, (513). Incomplete analyses have been published for several other derivatives (513, 524, 532).

possible for each sugar). Determinations of ring configuration by x-ray methods have been based primarily on considerations of the types of rings which seem best to fit the unit cell dimensions found for various derivatives and on comparison of actual and theoretical intensity data. Especially in the hands of Cox and co-workers (507–514) this approach has led to the conclusion that the pyranose ring is flat as far as the carbons are concerned, the oxygen lying outside the ring plane. For further discussion of such sterochemical problems, one is referred to an excellent review by Sponsler and Dore (515).

Important contributions to the x-ray interpretation of monosaccharides have also been made by Astbury and Marwick (516), Mark *et al.* (517, 518), Andress (519), Burgeini and Kratky (520), Sponsler and Dore (521), Marwick (522), McCrea (523), Young and Spiers (524) and Brackken *et al.* (525). The unit cell dimensions for various sugars, etc. are summarized in Table IV.

*Other compounds*

Among other compounds, those of the sterol groups have been the subject of particularly intensive inquiry on the part of Bernal and associates (533–535). Iball and Robertson (536) have also reported the unit cell dimensions of chrysene and 1 : 2 : 5 : 6-dibenzanthracene (a carcinogenic hydrocarbon) and Bernal (537) has published similar data for adenine and vitamin $B_1$. The sterol group is of great interest to biologists because it includes vitamin D, the male and female sex hormones, bile acids, toad poisons, and a variety of carcinogenic compounds (capable of inducing cancer in animals by external application, or by ingestion or injection). The studies of the sterol group have cleared up certain important questions of structure, for details of which one should refer to the papers cited. The published data are summarized in Table V.

Fatty acids, alcohols, and many other biochemically important compounds have also been subjected to x-ray analysis (307, 574–577), but space limitations forbid their consideration here.

*Tissues of organisms*

Many of the investigations previously discussed were carried out with intact tissues or

tissue fibers (e.g., in the investigations of silk, collagen, cellulose, etc.). We shall therefore consider here only researches not previously mentioned.

Sections from the epidermis, such as the skin of a cow's nose, show patterns typical of unstretched myosin and keratin, and if the tissue is stretched (e.g., under water) the patterns are of the $\beta$-type (542–545). Photographs obtained with nerve (547–551, 555, 578–581) are complicated by the variety of substance present, of which the lipoid content seems to contribute largely to the diffraction maxima observed.

An interesting investigation of the cell wall of the green alga *Valonia ventricosa* (552) showed that it is composed of successive layers in which the cellulose chains are inclined at large angles ($<90°$) to each other. X-ray studies of wood and lignin have been the subject of numerous reports (553, 554, 556, 557). The question of rubber structure (307) is particularly fascinating because of the intensive effort at the present time to develop artificial substitutes, but again one must forego consideration of the many contributions to this problem because of lack of space.

*Electron diffraction studies*

It would not be fitting to close this section without brief mention of structure investigations by electron diffraction methods. The technique has been applied primarily to the study of gases, for which it serves admirably. This field is the subject of a very recent review by Maxwell (558), and of earlier reviews by Brockway (559) and Beach (560). The technique can be applied equally well, however, to thin liquid and solid films. Fibroin, keratin and gelatin films (561), and films of oil, paraffin (562), and other biologically-important substances (563) have been investigated in this way.

TABLE V. *Summary of x-ray data regarding sterols, etc. All dimensions are in* A.

| SUBSTANCE | UNIT CELL DIMENSIONS | | | | CRYSTAL SYSTEM | SPACE GROUP | MOLS. PER UNIT CELL | PROBABLE MOL. LENGTH | REF.* |
|---|---|---|---|---|---|---|---|---|---|
| | $a$ | $b$ | $c$ | $\beta$ | | | | | |
| Phenanthrene | 8.66 | 6.11 | 19.24 | 98° | Monoclinic | $P2_1/c$ | | 9.62 | 533 |
| Retene | 8.54 | 6.25 | 23.4 | | Orthorhombic | $Aba$ | | 11.7 | 533 |
| Chrysene | 8.34 | 6.18 | 25.0 | 115.8° | Monoclinic | $P2/c$ | 4 | 11.4 | 533, 536 |
| Methylcholanthrene | 4.9 | 11.3 | 27.4 | 116° | Monoclinic | $P2_1/c$ | 2 | 13.7 | 533 |
| 1 : 2 : 5 :6-Dibenzanthracene | $7.84_b$ | $6.59_a$ | 14.17 | 103.5° | Monoclinic | $P2_1$ | | 13.75 | 533, 536 |
| Picene | 8.21 | 6.16 | 28.8 | | Orthorhombic | $Aba$ | | 14.4 | 533 |
| Cholesterylene | 15.85 | 7.66 | 19.25 | | Orthorhombic | $P2_12_12_1$ | | 20.5 | 533** |
| Cholestane | 11.2 | 11.0 | 19.8 | 104° | Monoclinic | $P2_1$ | 4 | 20.5 | 533** |
| Vitamin $B_1$ HCl | 12.62 | 20.53 | 6.96 | 66°5′ | Monoclinic | $P2_1/a$ | 4 | | 537 |
| Adenine HCl | 8.71 | 4.80 | 20.0 | 62°0′ | Monoclinic | $P2/c$ | $4+2H_2O$ | | 537 |
| Ergosterol·$H_2O$ | 9.92 | 7.57 | 38.5 | 115° | Monoclinic | $P2_1$ | 4 | | 534** |
| $\alpha$-Dihydroergosterol and ethylalcohol | 30.8 | 7.4 | 43.1 | 53° | Monoclinic | $C2$ | 12 | | 534 |
| Calciferol | 20.5 | 7.2 | 35.6 | 102° | Monoclinic | $P2_1$ | 8 | | 534** |
| Pyrocalciferol-calciferol | 20.2 | 7.35 | 35.4 | 100° | Monoclinic | $C2$ | 8 | | 534** |
| Lumisterol | 20.3 | 7.25 | 38.4 | 152.5° | Monoclinic | $P2_1$ | 4 | | 534** |
| Cholesterol | 15.8 | 19.1 | 74.0 | 112.5° | Triclinic | $P1$ | 32 | | 534** |
| Estrone (stable) | 16.28 | 7.46 | 12.15 | | Orthorhombic | $P2_12_12_1$ | 4 | | 535 |
| Estrone (unstable) | 9.9 | 7.7 | 18.2 | | Orthorhombic | $P2_12_12_1$ | 4 | | 535 |
| Estrone-monoclinic | 7.60 | 22.1 | 9.22 | 112° | Monoclinic | $P2_1$ | 4 | | 535 |
| Estradiol | 7.50 | 22.8 | 9.16 | 112° | Monoclinic | $P2_1$ | 4 | | 535 |
| Androsterone | 9.44 | 7.7 | 11.95 | 111° | Monoclinic | $P2_1$ | 2 | | 535** |
| Testosterone | 14.73 | 11.09 | 11.01 | 125° | Monoclinic | $P2_1$ | 4 | | 535 |
| $\alpha$-Progesterone | 10.27 | 13.88 | 12.31 | | Orthorhombic | $P2_12_12_1$ | 4 | | 535 |
| Pregnandiol | 10.2 | 7.3 | 24.6 | | Orthorhombic | $P2_12_12_1$(?) | 4 | | 535 |
| Pregnane | 12.0 | 6.29 | 22.6 | 100° | Monoclinic | $P2_1$ | 4 | | 535 |

* Other compounds reported in reference (533): Diels' $C_{18}H_{16}$ and $\gamma$-methyl-1 : 2-cyclopentenophenanthrene; Jacob's $C_{16}H_{14}$; "chrysene perhydrin," 2′ : 1′-naphtha-1 : 2-fluorene; Ruzicka's $C_{21}H_{16}$ from cholic acid; Ruzicka's $C_{25}H_{30}$ from gypsenogenin; 5-methyl-8-isopropyl-2′ : 1′-naphtha-1 : 2-fluorene; Diels' $C_{25}H_{24}$ from cholesterol; Ruzicka's $C_{26}H_{26}$ from ergosterol (stable and metastable forms); Ruzicka's $C_{27}H_{28}$ from phytosterols; 1 : 2-dimethyl phenanthrene; 1 : 2 : 7-trimethyl phenanthrene; 1 : 2-cyclopenteno phenanthrene (stable and unstable forms). Other compounds reported in reference (535): monobromethoxy estrone; oxyketone from dihydrocholesterol.

** Corrected to agree with the most recent unpublished results of Crowfoot, Bernal, and Fankuchen, through the kindness of Dr. I. Fankuchen.

### 3. Conclusions

In general, the most important contributions of the x-ray diffraction method to biology up to this time seem to have been those dealing with the structure of polymers, such as proteins, cellulose, etc. It is natural, however, that the trend should now be toward more precise evaluation of structural details, as in the application of Fourier analysis to the study of glycine etc. Probably more and more effort will be concentrated along this line in the coming years.

Real advances would seem to lie in the direction of the more precise study of fairly pure substances and compounds. Finally, the electron diffraction technique has proved its worth, even though but a beginning has been made in applying it. We may expect important contributions from its use in the future.

Several reviews have been listed in the preceding discussion. Additional ones by Astbury (564), Crowfoot (565), Bernal (566), Schmitt (567), Mitchell (568), Morse (569), Pichen (570) and Clark (571–573), should be mentioned.

## *III. APPLICATIONS OF INFRA-RED, RAMAN, VISIBLE AND ULTRAVIOLET SPECTROSCOPY TO BIOLOGICAL PROBLEMS*

We come now to the discussion of techniques which are useful in identifying the compounds present in, or isolated from, organisms and in determining details of their chemical structure. Of the techniques to be considered, ultraviolet and visible absorption spectroscopy have been the most extensively employed, partly because of their comparative ease of application, and partly because they lend themselves well to the purely empirical approach which has thus far proven most fruitful in applications of spectroscopy to biological and biochemical problems.

### A. Infra-Red and Raman Spectra

As is well known, the absorption of molecules in the far infra-red arises from rotational transitions, and in the near infra-red from combinations of vibrational and rotational transitions. From the quantum-mechanical expressions for the rotational and vibrational frequencies, the experimental spectra, and the atomic weights of the atoms involved, such quantities as the interatomic distances and bond strengths may be calculated for diatomic and simple polyatomic molecules. With increasing complexity of the molecule, the spectrum becomes more difficult of interpretation, because of the large number of possible vibrational and rotational energy changes.

Compounds of interest to the biochemist and biologist are, for the most part, highly complex; hence they are not suitable material for infra-red analysis by the usual methods applied to diatomic and simple polyatomic molecules. Another complication arises from the fact that many substances of biologic interest are solids which are water-soluble but not fat-soluble, and the high absorption of water in the near infra-red prevents their examination in dilute water solution.

Some workers, notably Coblentz, have applied a purely empirical approach to the infra-red absorption spectroscopy of biochemical materials in which (*a*) data have been collected and presented without analysis for what they may be worth in identifying compounds etc.; or (*b*) an attempt has been made on the basis of structural similarities to correlate the appearance of certain frequencies in the vibration-rotation absorption spectra with the presence of particular groups in the molecule.

The problem of the assignment of frequencies to particular groupings has been simplified by the accumulation of data regarding less complex molecules in the vapor state, in which such frequencies can be determined by calculations from bond strengths obtainable from other sources (thermochemical data, etc.), as well as by correlation of the appearance of certain frequencies with the occurrence of particular groups in related compounds. The utilization of this information in analyzing the spectra of complex molecules of biochemical interest is complicated, however, by the effects of constitution in causing shifts of the frequencies, as well as by the occur-

rence of numerous overtone and combination frequencies in addition to the fundamentals.

In Raman spectroscopy, the scattered radiation obtained from the substance when it is excited with monochromatic incident energy is examined for Raman lines of frequencies greater or less than that of the incident energy. Inasmuch as the changes in frequency on scattering found in Raman spectra are attributed to the effects of vibrational and rotational modes of the molecule, close correlation would be expected, and is found, between many Raman lines and the infra-red vibration-rotation frequencies for the same molecule. Hence the above remarks with regard to the assignment of frequencies to particular groupings apply as well to Raman as to infra-red spectra. There is this advantage of the Raman technique which should be emphasized—since the exciting and scattered radiation usually lie in the visible or ultraviolet regions, in which water is highly transparent, dilute water solutions may be employed without the difficulty caused by absorption encountered in the near infra-red.

On the whole, it may be said that infra-red and Raman spectroscopy of biochemically interesting materials are not in a very satisfactory state at the present time, and that the field needs to be developed. The value of these methods lies, at present, particularly in the investigation of special problems, such as those of association and hydrogen bonding, rather than in their use as a routine means of identifying unknown compounds or of investigating unknown structures, although the latter applications are frequently suggested in the literature.

The following discussion will be limited to compounds and substances of particular biologic interest.

## 1. Methods

*Infra-red*

The most usual infra-red spectrometers are commercial instruments similar to that described by Coblentz in 1914 (582). They are equipped with mirrors for collimating and focusing the radiant energy beam, and with interchangeable prisms for various regions of the spectrum, the approximate ranges being as follows: quartz, 1 to 3.5$\mu$; flourite, 1 to 7$\mu$; rocksalt, 7 to 15$\mu$; KBr, 12 to 22$\mu$. Coblentz (583) and Baly (584) have published extensive data regarding the optical properties of prism materials, and a recent paper by Barnes (585) discusses various alkali halides for infra-red prism use. Nernst glowers are ordinarily used as radiant energy sources. Pfund (587) has developed a source utilizing a sheet of ashed Welsbach mantle heated by an electric arc discharge and by gas. A thermopile (for example 588) and galvanometer (for example 589) may be used to measure the transmitted energy. Liquids and gases are examined in cells having windows of rocksalt, quartz, fluorite, etc., the transmission at each wave-length being compared with that of an optically-similar cell, empty, or containing solvent in case the substance under examination is in solution. The usual solvent for fatty substances is $CS_2$ (see Coblentz (583) for optical properties). Water solutions may be examined if the absorption by the water can be made sufficiently small by using highly concentrated solutions. Films of tissues and other solid substances may be employed if held rigid over an aperture, and certain solids may be deposited as thin films on plates of rocksalt, KBr, etc. (590). Various methods of wave-length calibration may be used—a convenient one being based on the prominent bands in ethyl and amyl alcohols (591). The spectra are usually plotted in terms of percentage transmission against wave-length.

Many papers have described useful equipment for infra-red spectroscopy (592–599). Of especial interest is the automatic recording infra-red spectrometer developed at Harvard (600).

*Raman*

In Raman spectroscopy, the problem is to excite scattered radiation characteristic of the substance by means of primary radiation which is, at the same time, as intense as practicable and nearly monochromatic; and to disperse the scattered radiation in a suitable spectrograph so that the Raman frequencies may be determined. The resulting spectrum shows not only the Raman lines but a broad line corresponding to the frequency of the incident radiation. The Raman frequencies, or Raman shifts, are the difference between the frequencies of the Raman lines and that of the exciting radiation.

$$\Delta\nu = \nu_I - \nu_R.$$

TABLE VI. *Raman and infra-red absorption bands corresponding to principal organic groups. Data from sources indicated.*

| GROUP | INFRA-RED BRODE (680), p. 221 | | RAMAN BRODE (680), p. 224 | | RAMAN HIBBEN (601), p. 22 | | RANGE OF RAMAN FREQUENCIES COMPILED FROM DATA IN HIBBEN (601) | | |
|---|---|---|---|---|---|---|---|---|---|
| | $\nu$ (CM$^{-1}$) | $\lambda(\mu)$ | $\nu$ (CM$^{-1}$) | $\lambda(\mu)$ | $\nu$ (CM$^{-1}$) | $\lambda(\mu)$ | FREQUENCY, CM$^{-1}$ | WAVE-LENGTH, $\mu$ | REMARKS |
| C–S | | | 645 | 15.51 | | | 630–745 | 15.85–13.45 | In thiocyanates and isocyanates: 629–690; in mercaptans: near 650; in thio ethers: near 733; in inorganic thio compounds: near 745. |
| C–N$^+$ | | | | | | | 750–1000 | 13.33–10.00 | |
| C–C Aliph. Arom. | 990 | 10.11 | 880–860<br>1580–1608 | 11.4 –11.6<br>6.32– 6.22 | 993 | 10.08 | 800–1100 | 12.50–9.09 | |
| C–N Nitro Amino | 1035 | 9.68 | 910–930<br>880 | 11.00–10.76<br>11.35 | 1033 | 9.70 | 830–1040 | 12.05–9.60 | |
| C–OH | | | 820–880 | 12.20–11.36 | 1030 | 9.70 | 835–1360 | 12.00–7.46 | Doublets at 1070 and 1090 in polybasic alcohols; approximately 1050 in simple alcohols. |
| N–N | | | | | | | 880–1050 | 11.35–9.53 | |
| N–O | | | 1000–1080 | 10.00–9.92 | | | 900–1000 | 11.11–10.00 | |
| C=N | | | 1650 | 6.06 | 1650 | 6.06 | 1460–1665 | 6.85–6.00 | Usually in region 1640–1655 in acyclic compounds. Conjugation with an ethylene group lowers the frequency. Frequency considerably less in cyclic compounds. Usually near 1575 in 6-member multiple-bond systems. |
| C=C Aromatic | 1630 | 6.14 | 1600–1650 | 6.25–6.06 | 1620 | 6.16 | 1500–1610 | 6.66–6.21 | In mono- and di-substituted benzenes: 1590–1610; in poly-substituted benzenes: 1500–1580. |
| C=C Aliphatic | | | | | | | 1600–1680 | 6.25–5.96 | Sharp, usually near 1640. In tri-substituted ethylenes, near 1675. |
| N=O | | | | | | | 1625–1665 | 6.15–6.00 | Near 1660 in aliphatic ketoximes. |
| C=O Acids | 1660 | 6.02 | 1654 | 6.05 | | | 1640–1740 | 6.10–5.76 | Approximately 1654 in simple acids; 1675–1740 in mono-substituted acids; two bands at 1740 and 1680 in di- and tri-substituted acids. |
| C=O Ketones | 1722 | 5.81 | 1710 | 5.84 | | | 1700–1710 | 5.88–5.84 | Lowered by presence of adjacent $NH_2$ groups to as low as 1606. |
| C=O Aldehydes | | | 1720 | 5.81 | 1700 | 5.89 | 1715–1740 | 5.82–5.75 | Formaldehyde is an exception, frequency 1768. |
| C=O Esters | | | 1720 | 5.81 | | | 1715–1780 | 5.82–5.62 | |
| C=O Anhydrides | | | 1750 | 5.71 | | | (a) 1745–1775<br>(b) 1804–1845 | (a) 5.73–5.68<br>(b) 5.54–5.42 | Two characteristic lines. |
| C–D | | | | | | | Approx. 2180 | Approx. 4.59 | |
| O–D | | | | | | | Approx. 2500 | Approx. 4.00 | |
| S–H | 2500 | 4.00 | 2470 | 4.03 | 2572 | 3.89 | 2570–2580 | 3.85–3.57 | Unique frequency in this region. |
| C–H Aliphatic | 2910 | 3.44 | 2920–2970 | 3.43–3.47 | 3050 | 3.28 | (a) 1450<br>(b) 2700–3300 | (a) 6.90<br>(b) 3.57–3.06 | |
| C–H Arom. | 3054 | 3.27 | 3054 | 3.27 | | | 3047–3070 | 3.29–3.26 | |
| O–H | 3380 | 2.96 | 3400 | 2.94 | 3650 | 2.74 | 3100–3650 | 3.23–2.74 | Broad bands. Near 3400 in mono- and poly-basic alcohols. |
| N–H | 3300 | 3.03 | | | 3370 | 2.97 | 3200–3400 | 3.13–2.94 | |

Mercury-arc primary radiation, filtered to make it approximately monochromatic, is ordinarily employed. The shorter wave-lengths excite more intense Raman lines. Hence the use of ultraviolet primary radiation, other factors being constant, has the advantage of shortening exposure times. The absorption of the sample for radiation of different wave-lengths must be considered in choosing exciting radiation, since high absorption results in low intensity of scattered radiation. Spectrographs of various types may be used, provided they have sufficient dispersion, good resolution, and sufficient speed (relative apertures in the range $f:3$ to $f:10$ are considered satisfactory).

These few remarks should suffice as an introduction to what follows, since the techniques have been reviewed in detail in numerous publications (see especially: 601–603).

## 2. Results

*Reviews*

For the infra-red, the early work has been reviewed by Coblentz (604). More recent books and reviews have been published by Lecomte (605), Czerny (606), Kellner (607), Matossi (608), Sutherland (609), Barnes and Bonner (610, 611), Sponer (612), Wu (613), and Rawlings and Taylor (614).

For Raman spectra, the excellent monograph by Hibben (601) surveys the literature up to June 1939. In addition, important reviews have appeared by Kohlrausch (615–617), Dadieu and Kohlrausch (602), and Martin (618).

*Assignment of frequencies to particular groups*

As pointed out in the introductory remarks, the systematic study of biochemical compounds by infra-red and Raman spectroscopy, as contrasted with the mere accumulation of empirical data, is based on the assignment of frequencies to particular molecular groupings. Table VI is a compilation of such data, taken principally from Hibben's monograph (601). It is customary to show in such tables only the usual frequencies found for the various groups in simple molecules. Table VI lists as well the range of frequencies reported for each group in various compounds, thus indicating the extent of the constitutive influence.

The following is a brief summary of investigations of various classes of compounds of biologic interest.

*Data with regard to compounds of biologic interest*

*a. Fatty acids and fats.*—The infra-red spectra of numerous fatty acids have been studied (619–627), especially with reference to association in formic and acetic acids and to the effects of substituting D for H in these acids. The C=O bond (about 1660 $cm^{-1}$) is prominent in the spectra of these acids, and shifts to higher frequencies when they are heated or diluted in water. Shifts of O–H (or O–D) frequencies are also found. This is taken to indicate association in the dimer form at the lower temperatures; the free monomer resulting when the acids are heated or put into solution.

Empirical data for various oils have been published by Coblentz (604, 628–630) and Vafiadi (625).

TABLE VII. *Prominent bands in infra-red absorption spectra of some simple sugars. Data of Barr and Chrisman (633) have been converted from wave-lengths to wave numbers ($cm^{-1}$).*

| SUGAR | FREQUENCIES ($CM^{-1}$) ATTRIBUTED TO VARIOUS GROUPS | | | UNASSIGNED FREQUENCIES ($CM^{-1}$) | |
|---|---|---|---|---|---|
| | DATA OF ROGERS AND WILLIAMS (632) | | | DATA OF ROGERS AND WILLIAMS (632) | DATA OF BARR AND CHRISMAN (633) |
| | $\nu$(C–H) $\nu$(O–H) | $\nu$(C=O) | $\delta(CH_2)$ | | |
| *d*-Arabinose | 3030 | 1700 | 1390 | 2040<br>1560<br>1220<br>1090 | |
| *l*-Arabinose | 3030 | 1700 | 1370 | 2040<br>1220<br>1110 | 4630<br>4240 |
| *l*-Xylose | 3030 | 1725 | 1350 | 2080<br>1100<br>960 | |
| *d*-Lyxose | 3030 | 1750 | 1350 | 2080<br>1590<br>1100 | |
| *d*-Mannose | 3030 | 1725 | 1350 | 2080<br>1040 | 4710<br>4260 |
| *d*-Galactose | 3030 | 1725 | 1370 | 2040<br>1590<br>1125 | 4610<br>4270 |
| *d*-Glucose (dextrose) | 3030 | 1670 | 1370 | 2080<br>1590<br>1100<br>1000 | 4650<br>4260 |
| β-Glucose | | | | | 4680<br>4240 |
| *d*-Levulose | 3030 | | 1350 | 1890<br>1100 | 4560<br>4220 |
| Maltose (hydrated) | | | | | 4670<br>4170 |
| *l*-Rhamnose (hydrated) | | | | | 4650<br>4260 |
| Sucrose (cane sugar) | | | | | 4610<br>4200 |
| *d*-Xylose | | | | | 4590<br>4220 |

*b. Hydrocarbons.*—Attempts have been made to use infra-red and Raman spectra for the analysis of mixtures of higher hydrocarbons (632, 633) and for the identification of various hydrocarbons (634–636), but the results are not impressive. Frequencies due to the C–H groups are prominent, and many other unassigned frequencies appear in the spectra.

*c. Carbohydrates.*—Infra-red data for a large number of monosaccharides have been published by Rogers and Williams (632) for the region beyond 3$\mu$ and by Barr and Chrisman (633) for the region 1.7 to 4.6$\mu$. These are summarized in Table VII. Barr and Chrisman (633) used thin films of the sugars, evaporated slowly on slides in such a way as to avoid crystallization. They could find no significant differences in the spectra caused by mutarotation or isomerism. Coblentz' early work includes infra-red data for simple sugars (604). Kutzner (634) found lines at 1180 $cm^{-1}$ and 1140 $cm^{-1}$ in concentrated aqueous solutions of six sugars, which he attributed to the keto and aldehyde forms. Ellis and Bath (635) used polarized light in the region 0.8 to 2.5$\mu$ to study the positions of OH groups. Raman data for sucrose (19 lines) have been published by Polara (636) and his results have been confirmed in general by Cleveland (637). The data favor the pyranose ring structure (compare discussion of x-ray studies of sugars, above). Other studies of sugars have been reported by Wulf, *et al.* (638, 639) and Khouvine and Arragon (640). For a detailed tabulation of Raman lines of various sugars see Hibben's monograph (601) page 150.

Among more complex carbohydrates and their derivatives empirical data have been published by Coblentz *et al.* for chitin (641), Cellophane (641), celluloid (642) and collodion (604).

*d. Amino acids and proteins.*—The publications of Edsall (643–648) regarding the Raman spectra of amino acids and related compounds are especially interesting. The C=O line, near 1700 $cm^{-1}$, appears in non-ionized amino acids (as it does in non-ionized fatty acids). The ionized acids, salts, etc. do not show it, but exhibit one or more lines near 1400 $cm^{-1}$, attributable to the $COO^-$ group. Lines due to $\equiv C-H$ or $=CH_2$ also appear in this region, as indicated by the fact that substitution of deuterium for hydrogen causes them to shift to the neighborhood of 1050 $cm^{-1}$. Near 3300 $cm^{-1}$, lines attributable to amino groups appear, unless these groups are ionized. If the amino groups are ionized their 3300 $cm^{-1}$ lines are replaced by lines in the 2500–3200 $cm^{-1}$ region. Edsall points out marked differences in the Raman spectra of isomeric amino acids, which should make their identification by Raman spectra possible. Some of Edsall's spectra are illustrated in Fig. 5.

As might be anticipated, the infra-red absorption of proteins seems to be essentially similar to those of the various amino acids of which they are constituted (649).

Gelatin has been studied extensively in the infra-red from the standpoint of the association of imbibed water with the gelatin molecule (650–652). In dried gelatin (650) assignable bands were found at 1.50$\mu$ (first overtone of NH and $NH_2$), 1.72$\mu$ (first overtone of CH, $CH_2$ and $CH_3$), 2.05 and 2.18$\mu$ (combination of fundamental valence and deformation vibrations of NH and $NH_2$),

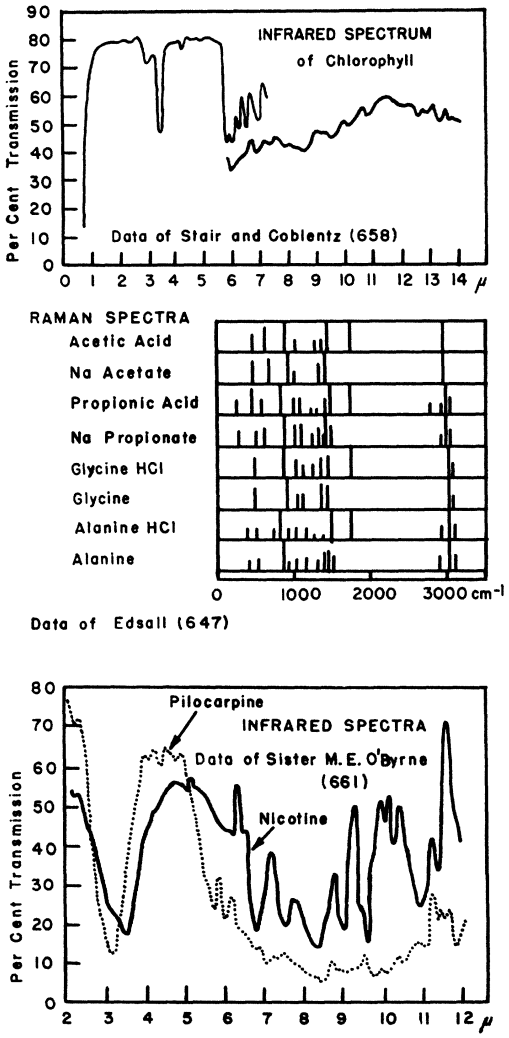



FIG. 5. Examples of infra-red and Raman spectra of substances of biologic interest.

and 2.28$\mu$ (C−H combination of fundamental and deformation vibrations). On addition of water, the 1.5$\mu$ band greatly diminished in intensity and the 2.05 and 2.18$\mu$ bands weakened. The 1.72 and 2.28$\mu$ bands did not change in intensity, but shifted slightly. Addition of $D_2O$ almost entirely suppressed the 1.5$\mu$ band.

Empirical data for egg albumin and for several natural tissues comprised mainly of proteins or chitin have been published by Stair and Coblentz (641) and by Rücher (653).

*e. Miscellaneous.*—Infra-red spectra from 500 to 1400 $cm^{-1}$ for several polycyclic hydrocarbons, including anthracene, $\beta$-methylcholanthrene, 2,3 benzanthracene and chrysene, have been reported by Lambert and Lecomte (654). From a study of the near infra-red spectrum of cholesterol, Stora and Freymann (655) concluded that this compound is associated in the molten state and is associated with the solvent in solution. During the era in which there was still considerable controversy about the structure of ergosterol and vitamin D, an interesting paper by Shelow (656) appeared on the near-infra-red absorption of ergosterol and its irradiation products which indicated that full ultraviolet leads to the formation of degradation products containing ketone groups while filtered ultraviolet ($\lambda > 2900$A) does not. Raman spectra of cholesterol and cholic acid from 500 to 3000 $cm^{-1}$ have been reported by Sannié (657). Labaune (631) has discussed the use of Raman spectra in the analysis of essential oils.

Numerous plant pigments have been studied. Stair and Coblentz (658) have published empirical data in the near infra-red for chlorophyll, xanthophyll, carotin, and other plant pigments. Katz and Wassink (659) claim that the infra-red spectra of chlorophyll and related pigments from algae and other unicellular organisms show shifts of the maxima when examined as extracts as compared with their examination in the intact cells. The use of Raman spectra for the identification of coumarins and chromones has been suggested (660).

Empirical data for several alkaloids in the region 1 to 12$\mu$ have been published by Sister M. E. O'Byrne (661). Frequencies corresponding to C−N, C−H, $C-H_2$ and benzene ring structures were found.

Studies of pentaerythritol and diketopiperazine with polarized near infra-red (662) are interesting because in the case of the former compound, the infra-red spectra indicated the structure to be different from that deduced from x-ray data, as discussed in a preceding section.

Various other compounds of biologic interest for which infra-red or Raman data have been reported include: pyridine (663), oximes (663), urea (663–665), glycine (664), guanidine (665), ascorbic acid (666, 667) and hemoglobin (668). The possible use of Raman spectra in the food industries has been discussed by Faure (669).

Of interest from the standpoint of the therapeutic use of infra-red radiation are Cartwright's (670) measurements of the infra-red transmission of the cheek. At $\lambda = 0.86\mu$, the transmission was 14 percent and at $\lambda = 1.15\mu$, 20 percent.

*Association and the hydrogen bond*

Probably the most fundamental investigations at the present time from the standpoint of biochemistry and biology are those dealing with the nature of association in biochemically-interesting compounds; in particular, with hydrogen bonds.

A hydrogen atom attached to a particular group may come within sufficiently close proximity to another radical, on the same or a different molecule, to be influenced by its force field, and in this way may form a rather loose association, or bond. The situation may be represented schematically by the formulae below, in which (I) represents an ordinary H-linkage (e.g., to carbon) and (II) a "hydrogen bond" type of association with a second group "X'." This, of course, does not imply a constant state such as

$$\text{(I) } X-H \qquad \text{(II) } X-H\cdots X'$$

that represented in (II) for all hydrogen bonds in the molecular complex, but rather a statistical effect which can be represented, on the average, by a loose bond $H\cdots X'$. Groups of the type C−H, N−H, and O−H all give rise to infra-red frequencies of the order of 3000–3500 $cm^{-1}$ (Table VI). Because of the relatively small mass of H, the vibration is practically that of the H atom toward and away from the "X" group. If a second group, "X'" should be loosely attached to H, a shift in frequency would be expected because of the restraining influence of this group

on the H vibrations. Thus, infra-red and Raman spectra should give indications of hydrogen bonding. In examining shifts in O−H frequencies in such compounds as fatty acids, their confusion with C−H frequencies can be avoided by substituting D for H in the O−H group, thus shifting the frequency of the unassociated group to lower values, as shown in the following tabulation.

*Frequencies of* O−H *and* O−D *in organic acids* (COOH *group*).

| Group | Not Associated | Associated |
|---|---|---|
| O−H | 3533 $cm^{-1}$ | 3080 $cm^{-1}$ |
| O−D | 2653 $cm^{-1}$ | 2300 $cm^{-1}$ |

Investigations of association in organic acids have already been referred to. Many other compounds have been studied. For details, one should consult the reviews by Sutherland (671), Fox and Martin (672) and Pauling (673), as well as numerous recent papers (586, 674–680). Discussions of hydrogen bonds in relation to the theory of protein structure have already been mentioned (356, 357, 441, 527). Infra-red investigations of hydrogen bonding in cellulose have been published by Ellis and Bath (681), and the possible role of H-bonds in starch structure has been discussed by Bawn, Hirst and Young (682). Pauling has postulated an interesting theory of antibody formation in which hydrogen bonds play an important role (683).

### 3. Conclusions

As stated in the introduction, the use of infra-red and Raman spectroscopy as an analytical tool in biochemical research may hardly be considered to be adequately developed. To your reviewer, this seems a natural consequence of the type of information which such spectra yield, and the technique seems more properly applied to the investigation of such special problems as hydrogen bonding than to the general identification of complex molecules.

Many interesting applications of infra-red and Raman spectroscopy to problems of molecular rearrangements in biochemical substances suggest themselves. For example, the amide-imidol type of tautomerism, to which your reviewer and his colleagues have largely attributed the influence of *p*H on the ultraviolet absorption of various purines and pyrimidines, might be investigated in this way (see discussion of ultraviolet spectroscopy, below). Many photochemical reactions in compounds of biologic interest, usually followed by ultraviolet absorption spectra, should be suitable subjects for infra-red and Raman analysis. When the physicist becomes better acquainted with biological and biochemical problems and the biological worker learns more of the terminology and techniques of the physicist, one may expect rapid progress in such investigations.

## B. Visible and Ultraviolet Absorption Spectra

Molecular absorption spectra in the visible and ultraviolet regions arise from electronic energy changes with which are combined, in general, vibrational and rotational energy changes. Each electronic transition gives rise to several bands corresponding to the various vibrational changes associated with it. The bands, in turn, are made up of lines corresponding to rotational transitions. In the spectra of certain diatomic gases, it is a comparatively simple matter to identify all of the band groups, bands, and lines. In polyatomic molecules, however, and especially in the complex molecules of interest to the biologist and biochemist, the number of possible electronic transitions (and associated vibrational and rotational transitions) is so great that the resulting spectra may be extremely confusing.

Furthermore, substances of biologic interest are usually examined in the solid or liquid state (including solids in solution), and the close packing of the molecules under such circumstances causes perturbing influences which result in broadening of the lines and bands into diffuse spectra. Frequently, the spectral region in which the substances are examined is one which leads to their photochemical transformation, and this gives rise to diffuse pre-dissociation or dissociation spectra. Finally, the techniques and equipment usually employed in absorption spectrophotometry are of insufficient precision and resolving power to show rotational* or vibrational struc-

* With regard to the rotational structure, it suffices to call attention to the fact that the usual spectrophotometric methods lead to curves defined by points 25 to 50A apart, whereas the separation of the rotational lines is of the order of 1A or less.

ture even if they might be observed by other means (e.g., a spectrogram made with an instrument of high dispersion and resolving power).

In considering visible and ultraviolet absorption spectroscopy as applied to biology and biochemistry, we usually have to deal, then, with data in the form of not-too-well-defined absorption curves which exhibit one or more broad maxima and which are not, in general, sufficiently informative with regard to vibration or rotational fine structure to permit of theoretical analysis. The data which have accumulated during the past forty years are mostly purely empirical, and of use only insofar as they represent reproducible characterizations of the compounds in question.

Some endeavor has been made to correlate structure and spectral characteristics by comparing the spectra of closely-related compounds, but no general, systematic basis for such correlation has been worked out as yet. Some attempt has also been made to determine the vibrational fine structure in particular compounds, and progress in this direction is slowly beginning to be made. It should be emphasized that the techniques used are as important a limitation in this regard as any other, and that with improvements in technical methods it may be expected that advances in the theoretical interpretation of the absorption spectra of biochemical substances will be more rapid.

Despite the above limitations, absorption spectroscopy has proved of great value in biological investigations, especially in: (a) the empirical identification of compounds available in quantities too minute to be examined by usual analytical methods; (b) the quantitative estimation of compounds with well-defined characteristic absorption; (*c*) the solution of structural problems by comparison of the absorption of an unknown substance with that of substances of known structure to which the unknown is suspected of being related; and (*d*) the study of biochemical reactions involving substances with characteristic absorption (e.g., in the living cell—biokinetics).

## 1. Methods

For a discussion of early techniques, one is referred to a report of the Optical Society of America Committee on Spectrophotometry (685) and to a paper by Baly *et al.* (686). Here only certain methods which are of general use or have been developed recently will be considered.

The substances investigated in absorption spectrophotometry are usually liquids or solids in solution. They are examined in suitable cells of glass (for the visible) or quartz (for the ultraviolet). A comparison cell is used to compensate for absorption by the cell windows and (if a solution is employed) by the solvent, and for reflections at the cell faces and walls. In the case of solid specimens, compensation for face reflections, internal scattering, and so forth, is somewhat more difficult, but may be accomplished approximately by the use of comparison specimens of transparent material (glass or quartz), or the effects of face reflections may be eliminated by measuring the transmissions $I_1$ and $I_2$ (where $I_1 < I_2$) of two different specimen thicknesses $t_1$ and $t_2$ (where $t_1 > t_2$) from which $I_1/I_2$ is the fraction of light transmitted by the thickness $t_1 - t_2$.

The essential units for absorption spectrophotometry are: (1) a suitable source of radiation; (2) a spectrometer, monochromator, or spectrograph for dispersing the radiation; and (3) a means of evaluating the relative intensities of the incident and transmitted energy at various wave-lengths (photometric method).

### *Sources of radiation*

General discussions of light sources useful in spectrophotometry have been published by Rompe (687) and others (688). For the visible region, incandescent lamps serve quite satisfactorily; a ribbon or small coil filament is used if a concentrated source is required. Pointolite lamps may also be employed. For the ultraviolet, the most usual sources are the condensed spark and the hydrogen arc. Other useful sources are underwater sparks, carbon arcs, and overloaded incandescent lamps with ultraviolet-transmitting windows.

*a. The condensed spark.*—This has been widely employed as a noncontinuous source for ultraviolet absorption spectrophotometry (689). It has the advantage of approximating a point source. Tungsten-steel electrodes yield a spectrum sufficiently rich in lines as far as 2000A for most investigations, but molybdenum electrodes

give a more nearly continuous spectrum. Electrodes $\frac{1}{4}$ to $\frac{3}{8}$ inch in diameter, spaced 3 to 4 mm apart, are customarily used, the spark being operated from a 10,000–20,000-volt, $\frac{1}{4}$- to $\frac{3}{4}$-kw transformer bridged by a condenser of about 0.005 mf capacity.

*b. Hydrogen discharge tubes.*—The discharge in hydrogen affords a continuous spectrum especially useful for analysis of fine structure in the ultraviolet. Its disadvantages for photometric work lie chiefly in the fluctuation and wandering of the region of high luminosity in most tubes, and in the fact that this region does not, ordinarily, approximate a point source. One of the earliest successful tubes was described by Bay and Steiner (690). Many tubes require continuous pumping, a disadvantage because of the complexity of the necessary accessory equipment. Some, such as the Hilger (689), which is a modification of Bay's and Steiner's, are sealed off. Most tubes operate at high voltages (691–693).

Smith (694) has examined the conditions for obtaining maximum intensity from the hydrogen discharge, and has developed a tube of high brilliancy. Almasy and Kortüm (695) have designed a tube for photometric work in which the region of high luminosity stays fairly well localized. Low voltage tubes have been described by Lemon (696), Freeman (697), Smith and Fowler (698), Duffendack and Manley (699), Munch (700) and Allen and Franklin (701). The last of these (701) is a sealed-off tube operated at 40 volts, 1 to 1.5 amperes, with an optimum hydrogen pressure of 5.5 mm, and a 3-mm hole in the cathode, providing approximately point illumination.

*c. The under-water spark.*—This provides a highly satisfactory continuous ultraviolet spectrum, but is noisy and must be cleaned frequently. Detailed specifications have been published by McNicholas (702) and Davis and Sheard (703). The spark, between tungsten electrodes separated by about 10 mm, is operated at high voltage from the secondary of a Tesla coil, the primary of which is excited by the condensed-spark discharge from a 2 to 3 kw, 15,000–20,000-volt transformer. The immersion water must be distilled carefully, and must be replaced constantly by a suitable continuous-flow arrangement, since it becomes contaminated with use.

*d. Other sources.*—Carbon arcs have been employed, but not extensively. They have high intrinsic brilliancy, and can be operated with fair constancy, but a large proportion of their energy is in the infra-red. Recently developed high pressure mercury arcs (704, 705) afford practically continuous radiation in the near ultraviolet and have very high surface luminosity. They have not come into general use in spectrophotometric work, but may be expected to find wider application in the future.

*Spectrographs, spectrometers and monochromators*

If but one spectrum is to be examined at a time, either a grating or a prism may be used as the dispersing device. If, however, (as is the more usual case in spectrophotometry) it is desired to examine simultaneously two or more spectra from adjacent beams focused on the spectrograph slit, a grating, as ordinarily mounted, cannot be employed. Gratings are not stigmatic, each point on the slit of a grating spectrograph gives rise to a line in the spectrum. Consequently, beams focused adjacent to each other on a grating-spectrograph slit produce overlapping spectra.

The many types of instruments used cannot be described here, but attention may be directed to two special points regarding prism devices: (1) For work with photoelectric cells in the ultraviolet, it is especially necessary to avoid scattered radiation since variation in the spectral sensitivity of the photo-cell may result in an exaggerated response to the stray light in comparison with that to the spectral region being investigated. Multiple-prism monochromators afford a solution to this problem. (2) While large, long-focus spectrographs produce large spectra in which the fine structure is more easily determined, they have the disadvantages of slowness when used for photographic photometry (since the spectrum is spread over a greater area of the plate) and of transmitting less short wave-length ultraviolet (2400 to 1800A), because of their greater optical paths in quartz. Your reviewer suggests from his experience that the focal length be chosen roughly according to the following expression: $f = 500/d$, where $f$ = focal length (in cm) required, and $d$ = the separation (in A) between the closest peaks or inflections which it is

desired to resolve at 2600A. Twyman and Allsopp (689, p. 61) recommend focal lengths about four times the values given by this expression.

*Photometric methods*

*a. Split-beam methods.*—These involve the employment of: (a) an optical system which splits the light into two beams, one of which passes through the solution and the other through the comparison cell, and then focuses the beams side by side on the spectrograph slit, and (b) a means of varying the intensity of the comparison beam, or of both beams. With such an arrangement (Fig. 6), two spectra appear adjacent to each other in the focal plane of the (prism) spectrograph, and by suitably adjusting the relative intensities of the beams, they may be made to match at any wave-length. Then, from the calibration and known characteristics of the intensity-adjusting device, one may determine the fraction of light transmitted or absorbed at the match point. The criterion of matching may be the eye, or a photoelectric device, applied directly or to the photographed spectra. In the ultraviolet region, the spectra must be photographed first, of course, before they can be matched visually.

Various methods are used for varying the relative intensities of the beams. In the visible region, polarizing and analyzing prisms, as in the König-Martens type of photometer, are frequently employed. The characteristics of such instruments have been discussed in detail by Gibson, McNicholas, and others (706–709). Adjustable vanes are used in the Judd-Lewis (710) instrument, and a rotating sector of adjustable aperture in the Hilger sector photometer and Keuffel and Esser photometer (684).

The rotating sector photometer has been used extensively for visible and especially for ultraviolet absorption spectrophotometry. The principal was first employed by Henri (711), and later developed by Twyman (see an early description by Howe, 712). Objections have been raised to its use because of the intermittency effect (the lack of equivalence of intermittent and continuous photographic exposures of equal total energy), but experience seems to show that this does not produce appreciable error (713–718). For further discussions of the intermittency

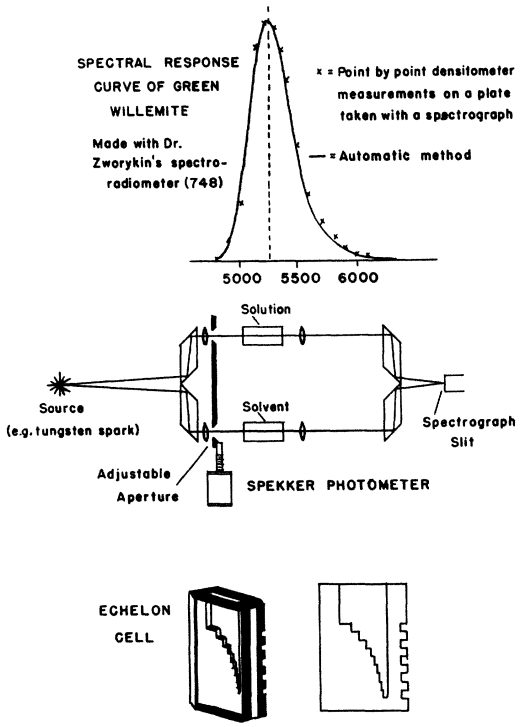



FIG. 6. Examples of spectrum methods and spectrophotodensitometer apparatus.

effect, one is referred to papers by Blair and Hylan (719) and by Webb (720). Webb found that above a critical flicker frequency, intermittent exposures were equivalent to continuous exposures of the same average intensity. Many modifications of the rotating sector photometer have been designed, among which may be mentioned Kortüm's (721) precision sector photometer for use in photoelectric spectrophotometry.

The "Spekker" photometer (722), employing a variable aperture to control the intensity of the comparison beam, was developed to overcome mechanical and optical difficulties inherent in the rotating sector type of instrument.

In all of the above methods, a separate setting is required for each percentage transmission (or density) to be determined. In order to speed up the procedure, methods have been devised for photographing several pairs of spectra simultaneously, as discussed below.

*b. Multiple spectrum split-beam devices.*—These include the notched echelon cell (723, 724), in which solutions of several different thicknesses are photographed simultaneously adjacent to

comparison spectra reduced in intensity by a rotating sector. In a sense, this device is a multiple Baly cell (725). Miller has suggested accomplishing a similar result with a multiple diaphragm (726), and O'Brien has used a toothed mirror in combination with a logarithmic sector (727).

*c. Applications of photo-cells to spectrophotometry.*—Photo-cells may be employed: (a) in conjunction with a monochromator to measure the intensities of the transmitted and comparison beams alternately at various wave-lengths, (b) to determine, by null methods, the match-point settings of a photometer such as the König-Martens, (c) to locate matched regions of photographed spectra, or to actuate a photodensitometer for tracing curves from photographed spectra, (d) to operate automatic or semiautomatic devices such as those described below, etc. General applications of photo-cells to spectrophotometry have been described by Gibson (728–730) and Sharp (731). Photoelectric methods have been used routinely for years for absorption spectrophotometry by various German workers (e.g., Pohl, Smakula, Kuhn, etc.), but have not come into general use in this country (for examples of typical American and English instruments, see references 732–740).

*d. Automatic methods.*—Among the most interesting recently developed spectrophotometric methods are those designed to be automatic or semiautomatic in operation.

Hardy's recording spectrophotometer for the visible region (741–744) has been in successful use for several years. It employs a principal described by Dobson and Prefect (745) and applied by them to spectrophotometry (746, 747) in which two beams of light are cut successively by a shutter (or other suitable device) so that they fall alternately on a photo-cell. The light chopper should provide a smooth transition from one beam to the other, so that if the beams are of equal intensities, no variation in photo-cell current results. If, on the other hand, they are of unequal intensities, fluctuations in photo-cell current are produced. These may be applied directly, or through an intermediate a.c. vacuum-tube amplifier, to a suitable indicating or recording device. In Hardy's instrument, the shutter is replaced by a pair of polarizing prisms, one of which rotates, and the alternating current derived from the amplified photo-cell-current fluctuations actuates a motor which rotates a third polarizing prism until a balance between the beams is obtained. This motor also drives a recording pen along the ordinate (relative absorption) axis of the record chart, so that the position of the pen always corresponds to the adjustment of the balancing prism. A separate motor changes the wave-length region selected by the monochromator and, at the same time, shifts the record chart to corresponding positions on its wave-length scale. Thus, the recording pen traces the curve of absorption (or reflection) automatically. About two minutes are required to trace an average curve.

Zworykin (748) has described an automatic recording spectroradiometer for registering the response of luminescent materials used as screens in cathode-ray tubes (see Fig. 6). It employs a chopped light beam acting on a photo-cell, the fluctuating current from which is amplified by an electron multiplier tube and an a.c. amplifier. The difference in voltage between the rectified output of the amplifier and that of a potentiometer actuates a motor which adjusts the potentiometer and drives the recording pen along the axis of abscissae until the potentiometer voltage is equal to that of the rectified signal. A second motor changes the spectral range transmitted by the monochromator and simultaneously moves the record chart along its wave-length axis. A particular feature of the instrument is its extreme sensitivity, achieved by the use of the electron multiplier tube, it being possible to obtain satisfactory records (in $1\frac{1}{4}$ to 5 minutes) from sources emitting 1 millilumen per square centimeter.

An instrument developed by Perkins (749) for measuring the relative sensitivity of photosensitive surfaces in different portions of the visible spectrum (4000–12,000A) traces curves in 30 seconds on a high persistence cathode-ray screen. With appropriate modifications, it has been used for absorption measurements.

Harrison's recording spectrophotometer (750, 751) uses a reflection grating. It covers the range from 2300 to 9800A (the limitation being imposed by the characteristics of light sources at present available). The light beam is passed alternately

through the absorption and comparison cells by an oscillating biprism, the intensity of the beam through the absorber being registered as a condenser charge until the comparison beam is matched to it by rotation of a variable diaphragm attached to the chart drum. When a match occurs, an electrical circuit is tripped and a point is marked on the record chart by a spark discharge. The method is a null one with regard to the measurement of intensities but not with regard to the variations of source intensity in the brief interval between the recording by the condenser of the intensity of the transmitted beam and the balancing of the comparison beam against this. Twenty points can be plotted per second, and a complete record requires from one to several minutes, depending on the spectral range covered.

Instantaneous cathode-ray methods have been suggested in two brief notes by Müller *et al.* (752, 753). The scheme consists essentially in sweeping a spectrum horizontally across a photocell slit in synchronism with the horizontal sweep of a cathode-ray oscillograph beam, and in applying the amplified photo-cell current to the vertical deflection plates of the oscillograph. The spectrum is thus traced automatically, and practically instantaneously, on the oscillograph screen. Your reviewer and his colleagues (754) have been working independently with such methods for some time past, using double photocells connected in push-pull to balance transmission and comparison spectra for absorption measurements, spectra recorded on loops of 35-mm motion picture film and run through a motion picture sound head connected through an amplifier to an oscillograph for rapid, semi-quantitative comparisons of emission spectra (e.g., from experimental ultraviolet sources), etc. While there are many inherent difficulties in such devices, the preliminary results seem to warrant further development.

*Methods for studying fine structure*

In instances in which the substance can be examined in the vapor state, the subsidiary peaks associated with vibration frequencies are most readily detected in this way. If liquids or solutions must be employed, low temperatures (e.g., liquid-air temperature) may be used to decrease the thermal motion of the molecules and the consequent number of molecular collisions, so that the subsidiary maxima associated with the vibration frequencies are made sharper and more distinct. Low temperature methods have been described by Kronenberger (755), Conant and Crawford (756), Arnold and Kistiakowsky (757) and others. Even at room temperature, a photodensitometer trace or careful visual examination of the plate sometimes reveals fine structure not evident in the usual spectrophotometric curve of the substance. The use of a source of continuous radiation, such as the hydrogen discharge tube, facilitates the observation of fine structure, but, contrary to the usual opinion, it is not absolutely essential (758).

By utilizing one or more of the above variations in technique, several workers have been able to detect fine structure in the spectra of amino acids, proteins, etc. (759–766). Prior to these publications, Heyroth and Loofbourow (767, 768) succeeded in demonstrating fine structure in the water-solution spectra of various purines and pyrimidines by using a match-point spectrum method in which variation in time of exposure was used to reduce the intensity of the comparison beam (771). Their success is probably accounted for by the fact that: (a) their time-variation method permitted the use of more highly concentrated solutions (which give greater contrast between maxima and minima) than may be used with, for example, the sector or Spekker photometers without unduly increasing the total exposure time required to photograph a complete plate; and (b) especial precautions were taken in the application of their method to insure great accuracy in the range of high extinction coefficients.

*Miscellaneous considerations*

*a. Sources of error in absorption spectrophotometry.*—The errors encountered in absorption studies and the necessary conditions for securing accuracy have been discussed by Miller (769), Twyman and Allsopp (689), Langstroth (770), Loofbourow (771), and Twyman and Lothian (772), among others. Attention is especially directed to the paper by Twyman and Lothian (772), which gives an excellent and concise survey of accuracies obtainable in practice. Several of

the papers previously referred to discuss precautions for obtaining the greatest accuracies with various spectrophotometric methods.

*b. Solvents.*—The usual solvents employed in the examination of solids in solution are: water, ethyl alcohol, methyl alcohol, diethyl ether, petroleum ether, chloroform, carbon tetrachloride, carbon disulphide, hexane and cyclohexane. The usual precautions with regard to purity of solvents suffice for visible absorption spectra, but for work in the ultraviolet, especially beyond 2500A, special methods of purification should be used. Water of sufficient transmittancy in the ultraviolet may be obtained by redistillation once or twice in an all-glass system. Alcohol may be purified by the method of Leighton, *et al.* (773). Methods for purifying methyl alcohol, ethyl alcohol, hexane, cyclohexane, carbon tetrachloride and chloroform are discussed by Twyman and Allsopp (689).

Consideration must be given to the effects of the solvent on the spectrum of the substance in question. As might be expected, polar materials yield different spectra when examined in polar and nonpolar solvents. Frequently, the *p*H of the solution affects the spectrum materially, as discussed below.

*c. Presentation of data.*—Some confusion arises from the variety of methods used for the presentation of absorption spectrum data. The following are the more common means of expressing the relative absorption at various wave-lengths (684, 689, 774, 775):

(1) Percent transmission:

$$T = 100(I_x/I_0),$$

where $I_0$ = intensity of incident radiation of wave-length $\lambda$,* $I_x$ = intensity of transmitted radiation of corresponding wave-length.

(2) Density (after Hurter and Driffield):

$$D = \log_{10} (I_0/I_x).$$

(3) Absorption coefficients: (a) For solids and liquids other than solutions:

$$a = (1/d) \log_e (I_0/I_x),$$

from Lambert's law, $I_x = I_0 e^{-ad}$ where $d$ = length of optical path through absorbing medium. (b) For solutions:

$$\alpha = (1/cd) \log_e (I_0/I_x),$$

from Beer's law, $I_x = I_0 e^{-\alpha cd}$ where $c$ = concentration.

(4) Extinction coefficients (after Bunsen and Roscoe): (a) For solids and liquids other than solutions:

$$K = (1/d) \log_{10} (I_0/I_x),$$

(b) For solutions:

$$k = (1/cd) \log_{10} (I_0/I_x).$$

(5) Molecular extinction coefficients:

$$\epsilon = (1/Cd) \log_{10} (I_0/I_x),$$

where $C$ = concentration in moles per liter.

(6) Logarithms of molecular extinction coefficients:

$$A = \log_{10} \epsilon.$$

(7) Absorption indices:

$$X = (\lambda/4d) \log_e (I_0/I_x),$$

where $\lambda$ = wave-length, in centimeters, in the substance under examination.

Of these, percent transmission (1) (or the related expressions, transmissivity, $t = I_x/I_0$, and opacity, $O = I_0/I_x$) is convenient for expressing the absorption of filters because it shows, at once, the fraction of the radiation transmitted at each wave-length by a particular filter. For other purposes, this mode of expression is less useful, because the conversion from one concentration to another involves the use of logarithms. Density values (2) have the advantage of being directly proportional to concentration (provided Beer's law holds) and to thickness (Lambert's law). Absorption (3) and extinction (4) coefficients, which are simply densities (or equivalent coefficients calculated with logarithms to the base "*e*") reduced to terms of unit thickness and concentration, are best suited to the general presentation of data, in which comparisons are

* It should be understood, of course, that "radiation of wave-length $\lambda$" implies radiation confined principally to a small range $\Delta\lambda$ from $\lambda'$ to $\lambda''$, where $\lambda' > \lambda$ and $\lambda'' < \lambda$. The range $\Delta\lambda$ and the distribution of intensity within that limited range, depends upon the resolving power of the spectrometer, the collimator slit width, the area of the spectrum selected for examination (e.g., by the exit slit of a photoelectric spectrophotometer), etc.

to be made between large numbers of substances. Absorption coefficients ($\log s_e$) are more generally used by German workers, and extinction coefficients ($\log s_{10}$) by English and American investigators, and this should be kept in mind in consulting the literature, as indication of the base of logarithms used is, unfortunately, frequently omitted in the presentation of data. In the identification of unknown substances, the molecular weight of the material in question is frequently not known, in which case molecular extinction coefficients (5) cannot be employed. The latter have their greatest usefulness in the calculation of the combined absorption of several molecular groups in a complex molecule, since it is usually merely necessary to add the molecular extinction coefficients of the constituent groups to obtain, very closely, those of the molecule as a whole. Thus, the coefficients of thymus nucleic acid (Fig. 9) are closely equal to the sums of the coefficients of adenine, guanine, thymine and cytosine (the contributions of the sugar and phosphoric acid groups to the absorption being negligible in comparison with that of the purines and pyrimidines). Logarithms of molecular extinction coefficients (6) are convenient if it is desired to compare the absorption of substances with widely different extinction values, or if it is desirable that the *shapes* of the absorption curves be independent of the units used for concentration or thickness. They have the disadvantage, however, of relatively compressing the variations in absorption at high extinction values, and of thus rendering less distinct the subsidiary maxima associated with particular vibration frequencies. Absorption indices (7) are mainly of theoretical importance, and are little used for substances of biologic interest.

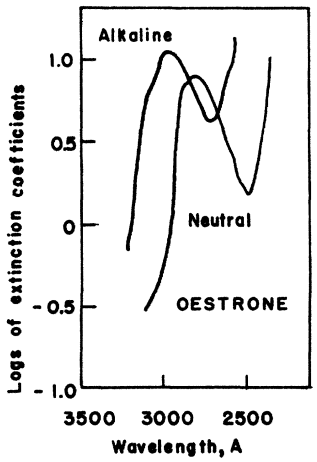



FIG. 7. Effect of *p*H on the ultraviolet absorption spectrum of oestrone, reference 792.

On the wave-length scale, wave-lengths in A are most usually employed for the ultraviolet and in A or m$\mu$ for the visible. Wave numbers ($\bar{\nu}=1/\lambda$, $cm^{-1}$) and frequencies (cycles per second, or Fresnel units, $f=(3\times10^5)/\lambda$, where $\lambda$ is in m$\mu$) have the advantage that harmonics of vibration frequencies are evenly spaced, but they have not come into general use (see Brode (684)).

*d. Calculation of the relative concentrations of constituents in mixtures.*—This may be accomplished by the solution of suitable simultaneous equations, derived from Beer's law (for details, see reference 689, p. 41).

*Reviews of methods*

In addition to the books and reviews already mentioned (684, 685, 689), Harrison (776) has published an excellent summary of methods in use up to 1934, and attention should also be directed to reviews by Gibson (777) of methods for the visible region and by Harvey (778) of photometric methods for the ultraviolet.

## 2. Results

The number of papers containing useful absorption spectrum data is so great that the mere listing of them would unduly lengthen the bibliography. It will be necessary, therefore, to limit this discussion to a brief survey of what has been done.

*Correlation of chemical structure and spectra*

To a close degree of approximation, electronic transitions associated with unsaturated linkages give rise to absorption bands in the visible and ultraviolet from about 2000 to 8000A,† whereas those associated with saturated linkages give rise to bands only in the far-ultraviolet, Schumann region ($\lambda<2000$A). Rather broad generalizations as to the groupings responsible for absorption have been made for many classes of compounds; in particular for dyestuffs, in which instance the

† It was suggested by Nietzki, reference 780, as early as 1879 that color is related to unsaturation.

organic chemist designates such groups as "chromophores" (see Brode (684), Cohen (781), Förster (797), Niimiya (798), Beilenson *et al.* (811), and Pruckner and Stern (812) for discussions of the absorption of dyes).

As discussed previously, one would expect the absorption associated with an electronic transition in a particular group to show, under appropriate conditions, subsidiary maxima associated with vibrational energy changes. Such subsidiary maxima have been observed, for example, in the ultraviolet absorption spectrum of solutions of benzene (782–784), but the identification with particular chromophores of particular bands in the spectra of most molecules is complicated by the influence of constitution on the positions of the subsidiary maxima.

The following generalizations may be made with regard to absorption in the region 2000–8000A:

(1) The principal groupings contributing bands to the absorption spectra of organic molecules are (684, 796, 818, etc.):

$$>C=C<,\quad >C=O,\quad >C=S,\quad >C=N-,$$

$$-N=N-,\quad -N=O,\quad >S=O,$$

$$-N\begin{matrix}\diagup\!\!\diagup O\\ \diagdown\!\!\diagdown O\end{matrix},\quad -N=N-,\quad >C=C=O,$$

and conjugated chains or rings made up of $>C=C<$, $>C=N-$, or $-N=N-$ groups.

(2) The frequency of the absorption maximum associated with a particular group is decreased ($\lambda$ increased) when substituents are attached to it (785, 813). This is often called the "weighting" effect.

(3) Single (saturated) linkages tend to isolate absorbing groups from each other and to nullify their effects on one another, so that the composite absorption curve becomes simply the sum of the bands due to individual absorbers. A separation of groups by two or more single linkages is sufficient to insure their approximate independence (757, 813).

(4) In conjugated-double-bond linkages

$$(-C=C-C=C-),$$

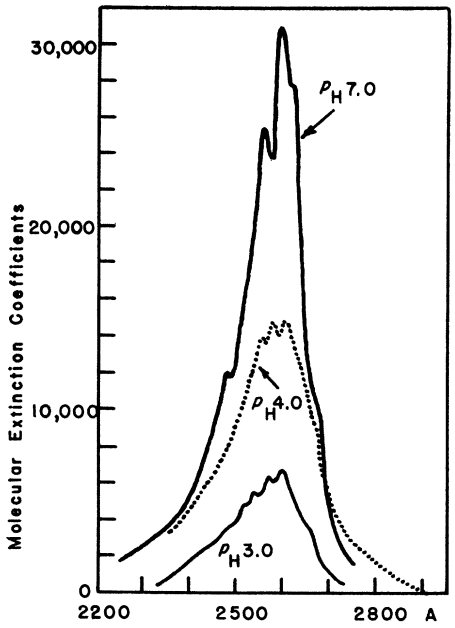



FIG. 8. Effect of *p*H on the ultraviolet absorption spectrum of barbituric acid. Data of Loofbourow and Stimson, reference 802.

the coupling of unsaturated groups is sufficiently strong to cause the effect of any one group to extend for some distance along the chain, or ring. Consequently, an increased number of conjugated double bonds results in a shifting toward lower frequencies (longer wave-lengths) of the bands associated with all the groups in the system; i.e., a general shifting of the composite absorption spectrum (787–790).

(5) "Weighting" of an absorbing group, either by direct substituents or by distant groups coupled through conjugated linkages, results in increased absorption (785, 787–790, 814).

(6) Changes in "weighting" caused by salt formation (792–794), association (829, 830), etc., or changes in kind and position of unsaturated groups due to molecular rearrangements (tautomerism) (767, 768, 795, 800–803, 941) produce corresponding changes in the absorption spectra. The changes in spectra caused by salt formation are well illustrated by the increase in absorption and shift of the maximum of oestrone* from about

* This was attributed to enolization by Pedersen, *et al.*, reference 791, but has been shown by Callow, reference 792, to be caused by salt formation at the phenolic-3-hydroxy group, an effect analogous to similar changes in phenols, references 793, 794.

TABLE VIII. *Position and extinction of absorption maximum. Data from Scheibe and Frömel (796).*

| | Lowest λ of Substances Listed | | | Highest λ of Substances Listed | | |
|---|---|---|---|---|---|---|
| Group | Substance | λ | ε | Substance | λ | ε |
| C=O | Acetone $CH_3 \cdot CO \cdot CH_3$ | 2795 | 15 | Tetraphenylcyclopentadienone | 5100 | 1000 |
| C=C | Trimethylethylene $CH_3-C(H)=C(CH_3)_2$ | 1850 | *ca.* 10,000 | Decatriene-3,5,7-oic acid $CH_3-CH_2-C(H)=C(H)-C(H)=C(H)-C(H)=C(H)-CH_2 \cdot COOH$ | 2650 | 43,000 |

2800A, in acid or neutral solution to about 2950A in alkaline solution (792). The influence of molecular rearrangements accompanying *p*H change may be illustrated by the effect of the reaction (Fig. 8) on the absorption spectrum of barbituric acid (802). It is principally to such actions that the effects of solvents and *p*H on absorption spectra are to be attributed. The influence of the reaction on absorption is, of course, the basis of the colorimetric determination of *p*H (see 684, also 824–828).

An excellent summary of these matters is given in a review by Scheibe and Frömel (796). The extent of the shift in the absorption bands associated with particular groups when they are influenced by constitutional effects is shown in Table VIII.

The spectra of benzene and benzene derivatives have been subjected to detailed electronic and vibrational analysis, using both vapors and solutions (804–808), and these analyses have been used in interpreting the absorption of other compounds (809). Seshan (810) investigated the influence of the physical state on the absorption and fluorescence of organic compounds and found on progression from vapor→solution→liquid state→solid state a progressive broadening of the absorption bands and shifting toward the red. Bandow (907, 924) has published studies of the spectra of sulphuric-acid solutions of many compounds of biochemical interest. Analysis of the electronic transitions involved in the absorption of various organic compounds and other discussions of theoretical interest have been published

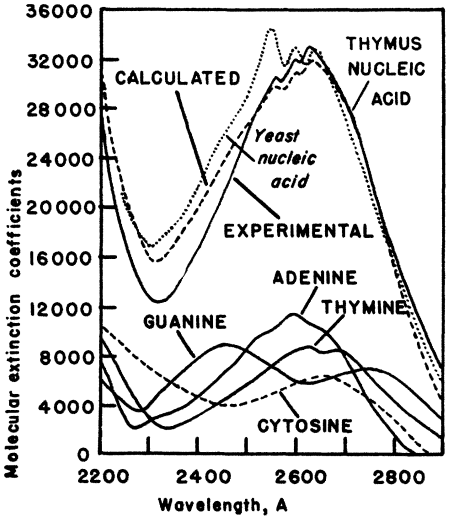



FIG. 9. Equivalence of the ultraviolet absorption spectrum of thymus nucleic acid and the sum of the absorption spectra of its purine and pyrimidine constituents, references 875 and 881.

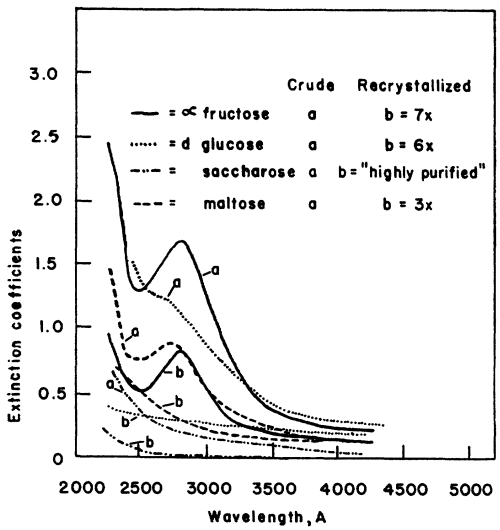



FIG. 10. Ultraviolet absorption spectra of certain carbohydrates, references 841 and 842.

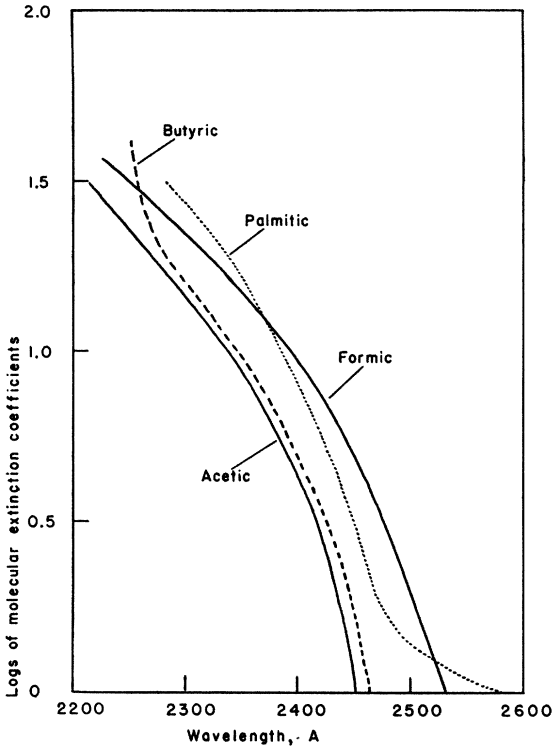



FIG. 11. Ultraviolet absorption spectra of fatty acids, after Mme. Ramart-Lucas, *et al.*, reference 848.

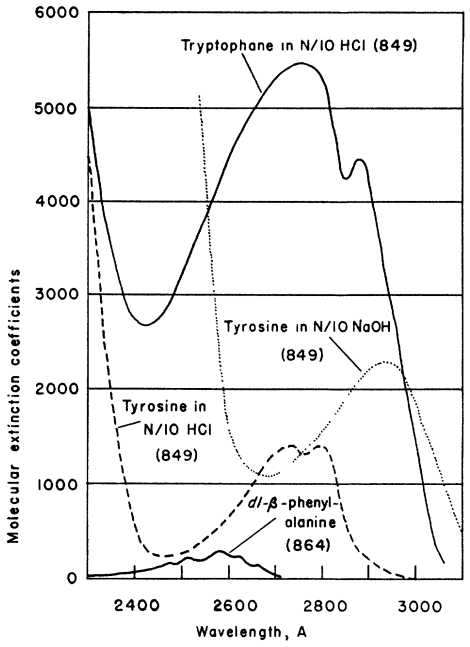



FIG. 12. Ultraviolet absorption spectra of aromatic amino acids.

in recent papers by Duschinsky (815), Kortüm (816), Burawoy (817), Kato *et al.* (818, 819), Heyfield (820), Dimroth (821), Chako (822), Sklar (823), and Smakula (831).

*Data regarding substances of biochemical interest*

*Reviews and tables.*—The extensive tabulated data of Henri (832), Carr *et al.* (833) and Ellinger (834) are quite useful for quantitative absorption measurements of individual compounds. Bibliographies by Walker (835) and others (836) are convenient sources of literature references and the excellent recent book by Miller (837) contains much useful information concerning the absorption of compounds of biologic interest. Other reviews and books include those of Gillam (838), Brode (684) and Lavin (839). Absorption spectroscopy in relation to vitamins and hormones has been reviewed by Morton (840) and in the case of vitamin D, by Loofbourow (774, 779).

*Data for specific substances.*—The general characteristics of the absorption spectra of carbohydrates, fatty acids, amino acids, proteins, purines and pyrimidines, nucleic acids, and polycyclic hydrocarbons are summarized in Table IX and Figs. 7–14. Investigations of fine structure in the spectra of amino acids and proteins were referred to in the discussion of methods above. Table X lists some of these data.

Certain other results may be mentioned briefly. Among vitamins, the spectrum of $B_1$ was the subject of controversy until Williams *et al.* established this vitamin's structure. Morton's review (840) leaves open the question of the validity of the 2470A maximum of Peters and Philpot (882) and Holiday (883) or the absorption near 2600A correlated by Heyroth and Loofbourow (874, 881, 884) with $B_1$ activity. Ohdake (928) found bands at 2390 and 2680A and Heyroth and Loofbourow (885) published spectra of highly purified preparations showing maxima at 2350 and 2680A and a minimum at 2500A, from which they concluded that $B_1$ contains a pyrimidine similar to cytosine. The spectra of Williams and co-workers (886) confirmed quantitatively these last data (885) and the elucidation of $B_1$ structure by Williams *et al.* has established the

TABLE IX. *General characteristics of absorption spectra of selected substances of biochemical interest in the region* $\lambda = 2000\text{–}8000A$.

| SUBSTANCES | ABSORPTION 2000–8000A<br>$e$ = MOLECULAR EXTINCTION COEFFICIENT | REMARKS | REF. |
|---|---|---|---|
| *I. Carbohydrates* | | | |
| Arabinose, xylose, galactose, glucose, maltose, lactose, sucrose, raffinose. | End absorption only, rising rapidly from about 2300A toward shorter wave-lengths. | Commercial sugars yield an absorption maximum at about 2700–2800A, with a minimum at about 2450A. On repeated recrystallization, the 2700–2800 band disappears (except possibly in the case of fructose and sorbose) and only end absorption remains. | (841–847) |
| Fructose<br>Sorbose | Max. 2800A, min. 2475A \| $e$ = extremely low, less than 10.<br>Max. 2750A, min. 2420A / | | |
| *II. Fatty acids* | | | |
| A. Saturated (formic, acetic, butyric, hexanoic, octanic, myristic, palmitic) | End absorption only, rising rapidly from about 2500A toward shorter wave-lengths. | | (848) |
| B. Unsaturated, conjugated. | Single broad absorption band increasing in extinction and shifting to longer wave-lengths with increased conjugation. | Bands vary in position from about 2100 to 3000A, $e$ = of the order of 25,000 to 50,000. | (787–788) |
| C. Unsaturated, not conjugated (e.g., oleic, linolic, linoleic). | Separate subsidiary maximum usually present for each >C=C< linkage. | | (684 p. 136) |
| *III. Amino acids* | | | |
| Tyrosine | In acid solution:<br>Max. 2750–2800A, $e$ = 1300–1600.<br>Min. 2450–2500A, $e$ = 200.<br>In alkaline solution:<br>Max. 2850–2900A, $e$ = 2200.<br>Min. 2250A, $e$ = 1200. | Also secondary maxima and minima, the number of which observed depends on the method used. For fine structure, see Table X. | (760–763, 849–869) |
| Tryptophan | Max. 2750–2800A, $e$ = 5100–6000.<br>Min. 2425–2460A, $e$ = 1000–2800. | For fine structure, see Table X. Not appreciably affected by $p$H, but see (849). | |
| Phenylalanine | Central max. 2560–2575A, $e$ = 100–180.<br>Min. 2340A, $e$ = 28. | Many prominent subsidiary maxima. For fine structure, see Table X. Not appreciably affected by $p$H. | |
| Other amino acids, not containing an aromatic nucleus. | End absorption only, rising from about 2500–2200A toward shorter wave-lengths. | Selective absorption in the region $\lambda > 2000A$ is to be associated with the presence of an aromatic nucleus. | |
| *IV. Proteins* | Their absorption is attributable to their amino-acid content, and is therefore principally due to tyrosine and tryptophane and, to a lesser extent, to phenylalanine. | In the case of nucleoproteins, the absorption of the nucleic acids contributes materially to the spectrum. | (700, 761, 763, 851–854, 870–873) |
| *V. Purines and pyrimidines* | | | |
| Adenine | $p$H 5.0 Max. 2625A, $e$ = 13,200.<br>Min. 2300A, $e$ = 2500. | Spectrum changes with $p$H according to (874) but not according to (941). | (767, 768, 795, 802, 875, 878, 879, 933, 941–945) |
| Guanine | $p$H 6.5 Max. 2460A, $e$ = 9000,<br>and 2730A, $e$ = 7500.<br>Min. 2280A, $e$ = 3500,<br>and 2625A, $e$ = 6000. | Spectrum changes markedly with change in $p$H (768, 941). | |
| Uracil | Unbuffered<br>Max. 2600A, $e$ = 9000.<br>Min. 2290A, $e$ = 1700. | Spectrum changes markedly with change in $p$H (767). | |
| Thymine | Unbuffered<br>Max. 2625A, $e$ = 8600.<br>Min. 2330A, $e$ = 2000. | Spectrum changes with $p$H (875). | |
| Cytosine | Unbuffered<br>Max. 2650A, $e$ = 6200.<br>Min. 2475A, $e$ = 4300. | Spectrum changes with $p$H (875). | |
| Other purines and pyrimidines | Similar characteristic absorption in the region 2400–2700A. | The influence of $p$H on absorption of the above compounds has been attributed to tautomeric rearrangements (768, 795, 875). | |
| Nucleosides and nucleotides | Their absorption is attributable to the purine or pyrimidine portion of the molecule, the contribution of sugar and phosphoric acid being negligible. | | (768, 935–940) |
| *VI. Nucleic acids* | The absorption of nucleic acids is mainly attributable to the purines and pyrimidines they contain (Fig. 9). Nucleic acids in general have a broad maximum at 2600A, $e$ = *ca.* 30,000, and a minimum at 2300A, $e$ = *ca.* 12,000–15,000. | The spectra are, to a close approximation, the sums of the spectra of the constituent purines and pyrimidines at the $p$H in question. | (767, 875, 876–878, 881) |
| *VII. Polycyclic hydrocarbons* | Characterized by broad, complex bands, with many well-defined subsidiary maxima, in the region 2000–3700A. Max. $e$ of the order of 5000 to 100,000. | The absorption may be attributed to the resonators in the aromatic nuclei of which these compounds are constituted. | (783, 784, 792, 840, 880) |

fact that one ring of the molecule is a pyrimidine of the cytosine type. Ultraviolet absorption spectra played so important a role in the elucidation of the vitamin D problem that the literature is too extensive to consider here, and one is referred to previously-mentioned reviews (774, 779, 840). An excellent summary of recent work on other vitamins will be found in Miller's book (837).

In an extremely interesting series of investigations, Caspersson *et al.* found that rapidly-dividing cells of yeast (878) and animal tissues (887) show greater 2600A absorption, attributed to higher nucleic acid content, than resting cells. Their work has been confirmed for yeast by Landen and Uber (888). Its significance lies in its indication of the importance of nucleic-acid-like substances in the processes of cell division.

Spectra of the porphyrins have been extensively studied (889–893, 914), particularly by Stern *et al.* (894–896), as have also spectra of the plant pigments chlorophyll, xanthophyll and the carotenoids (799, 897–906, 916), and of the bile pigment bilirubin and related compounds (786, 867, 908–915). The absorption of blood serum has been shown to be caused principally by its tyrosine and tryptophane content (854). Many studies of the spectra of blood serum and other blood constituents have been published (917–923), in some of which an endeavor has been made, with results which are at present controversial, to utilize changes in such spectra in the diagnosis of pathologic conditions.

Studies of the absorption of visual purple (737, 925–927, 929) have helped to establish the role of vitamin A in night and twilight vision, and thus have paved the way for the lessening of night motoring and airplane-landing accidents caused by faulty regeneration of visual purple associated with vitamin A deficiency. Because of the importance of the accurate determination of the hemoglobin content of the blood in medical diagnosis and of the essential role of hemoglobin in the life of the animal, developments in the application of spectrographic technique to hemoglobin estimation and to the study of its structure and that of its derivatives are of great interest (930–932).

Absorption spectrophotometry has played an important role in elucidating the structure of respiratory enzymes and in following their activities in the living cell (biokinetics). The respiratory enzyme complex called "cytochrome" which, through its ability to be reversibly oxidized and reduced, acts as a hydrogen carrier in the oxidative processes taking place in the living cell, has a characteristic absorption spectrum in the reduced form consisting of a series of bands in the visible region of the spectrum (933). On oxidation, the bands near 6000A fade. These spectrographic changes can be observed in living

TABLE X. *Fine structure in spectra of amino acids and proteins.*

| SUBSTANCE | REF. | ABSORPTION BANDS | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tyrosin<br>Tryptophane<br>Phenylalanine | 763 | 2366 | 2418 | 2466 | 2517 | 2574 | 2606 | 2635 | 2671 | 2672<br>2694<br>2714 | 2747 | 2794 | 2816 | | 2888 |
| Pepsin | 761 | | | | 2520 | 2580 | | | 2650 | 2690 | 2760 | | 2850 | | 2900 |
| Tobacco mosaic virus | 766 | | | | | 2550 | 2600 | | 2660 | 2690 | 2750 | | broad band 2820 | | 2910 |
| Serum albumin | | | | | 2533 | 2583 | 2613 | 2645 | | 2688 | 2733 | 2788 | 2847 | | 2900 |
| Egg albumen | | | | | 2532 | 2587 | | 2650 | | 2680 | 2742 | 2799 | 2855 | | 2923 |
| Thyroglobulin | | | | | 2534 | 2581 | 2614 | 2645 | | 2682 | 2743 | 2796 | 2841 | | 2909 |
| Euglobulin | | | | 2487 | 2529 | | 2616 | 2640 | | 2680 | 2749 | 2795 | 2849 | | 2915 |
| Pseudoglobulin | 763 | | | | | 2587 | | 2649 | | 2691 | 2747 | 2794 | 2849 | | 2916 |
| Pneumococcus antibody | | | | | | 2591 | | 2649 | | 2685 | 2768 | | 2850 | | 2911 |
| Gelatin | | | | | 2529 | 2584 | | 2644 | | 2679 | 2745 | | 2839 | | |
| Insulin | | | | | 2530 | 2586 | | 2645 | | 2683 | 2766 | | 2839 | | 2898 |

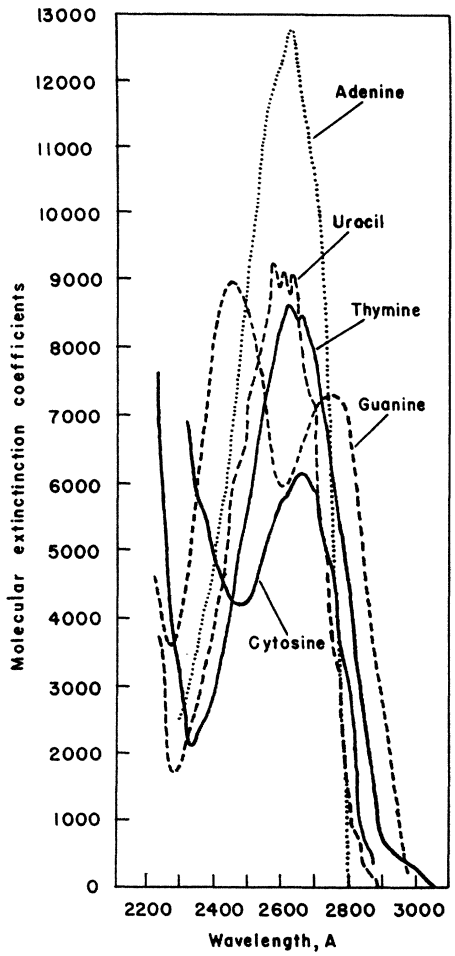



FIG. 13. Ultraviolet absorption spectra of certain purines and pyrimidines, references 767, 768 and 795.

cells and used to follow oxidative changes within the cell under various conditions. For example, a suspension of yeast in an $N_2$ atmosphere exhibits the typical reduced-cytochrome bands, but if oxygen is bubbled through the suspension, they fade. Similar changes are observed in animal tissues. In insects (e.g., wax moths) bands indicating reduced cytochrome appear after vigorous exertion, but these disappear, indicating oxidation of the cytochrome, if the insect remains quiet.

The coenzymes diphosophopyridine nucleotide and triphosphopyridine nucleotide (933, 934) both have a band at 2600A attributable to their pyrimidine portion. In the reduced form, they exhibit a band near 3400A and a white ultraviolet fluorescence, both of which disappear on oxidation. These properties can be used as a means of identification and of quantitative estimation.

### 3. Conclusions

The usefulness of visible and ultraviolet absorption spectrophotometry as an empirical means of identifying biochemical substances is well established. Its application to the identification of unknown structures by means of quantitative relationships between structure and absorption rests, at present, on less firm ground, but progress is rapidly being made in that direction.

To your reviewer, the most interesting trends at present are those toward the interpretation of visible and ultraviolet absorption spectra in terms of electronic and vibrational analysis. Herein seems to lie the best possibility of establishing visible and ultraviolet absorption spectrophotometry as an invaluable analytical tool in biochemistry and in the field of organic chemistry generally. In the realization of this possibility, it is to be expected that the absorption spectrophotometry of organic compounds will be extended to the ultraviolet region of $\lambda < 2000$A, in which the contribution of unsaturated groups to absorption is important. Reference to this region of the spectrum has been omitted in the preceding discussions because technical difficulties have thus far so greatly limited progress that only a few papers have appeared.

## C. Fluorescence Spectra

Applications of fluorescence spectroscopy to problems in biochemistry are discussed in detail in a recent monograph by Dhéré (946), and will be considered only very briefly here.

The method consists in exciting the visible or infra-red fluorescence of the substance in question by radiation from an ultraviolet source, suitably filtered (e.g., by "Wood's glass") to eliminate as much as possible of the visible radiation, and the observation of the fluorescent light, either visually, photoelectrically, or by the aid of a spectrograph.

In visual observation, qualitative estimates are made of the color and intensity of the fluorescent light. Photoelectric measurements have

the advantage of greater quantitative precision, but require careful secondary filtering of the fluorescent light to prevent transmitted or scattered primary ultraviolet radiation from entering and affecting the photo-cell, and one must resort to visual observation, or to the use of suitable color filters, for qualitative estimates of color. Spectrographic analysis of the fluorescent radiation permits the color spectrum to be recorded quantitatively. Even then, however, the problem of presenting reproducible data is not as simple as in absorption spectrophotometry, because the spectral energy distribution of the fluorescent light is dependent upon the characteristics of the exciting radiation. The majority of reports in the literature deal with qualitative, visual observations.

In addition to Dhéré's monograph (946), a book by Radley and Grant (947) and general discussions by Blorhenalsev (948), Weiss (949), and Dhéré (950) will be found to be useful sources of information.

Among the most dramatic results which have come from application of the technique are the identification by Mayneord, Hieger, Kennaway, and others, of the active agent in cancer-producing tars as a polycyclic hydrocarbon, on the basis of similarities in the fluorescent spectra of cancer-producing tar fractions and that of 1-2 benzanthracene (951–953). This and later work (954–959) lies at the basis of the present knowledge of carcinogenic hydrocarbons with the phenanthrenecyclopentane nucleus, as well as of related carcinogens. Developments from these investigations have not only pointed the way to the control of the cancer hazard among coal-tar workers and chimney sweeps, but have also provided the cancer investigator with a means of producing malignant tumors routinely in laboratory animals.

Of other substances, the fluorescence of porphyrins (960–963) and of chlorophyll (964, 889, 890) have been studied extensively. The significance of the latter work will be considered in the discussion of photosynthesis in a later part of the review. Recent papers by Ingold and Wilson (967, 968), Krishnan and Seshan (969), Finkelburg (970), Pringsheim (971) and Lewschin (965, 966) discuss theoretical aspects of fluorescence, and Minibeck (972) has published several interesting papers on applications of fluorescence to microanalysis.

### D. Emission Spectra

The principal application of emission spectra to biological problems has been in the detection of metals in tissues, for example, in studying the accumulation of lead and other metals in persons subjected to them. The techniques employed are the standard ones familiar to physicists (977), with slight modification to permit the use of biological samples (973).

The field has been reviewed in detail by Gerlach and Gerlach (973). The method has received application in the determination of the metallic nutritional requirements of yeast by Richards and Troutman (974). Armstrong and Brackett (975) have discussed the spectrographic analysis of biological fluids for heavy metals, and Zimmer (976) has investigated the magnesium content of the blood in various diseases.

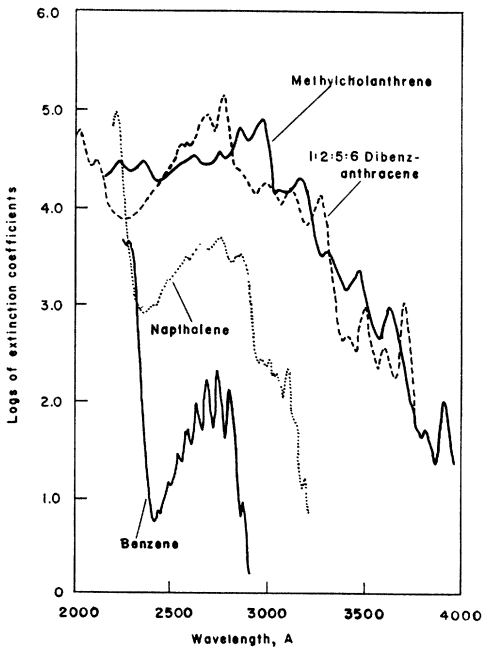



Fig. 14. Typical ultraviolet absorption spectra of policyclic hydrocarbons. Data of Mayneord and Roe, reference 783.

## IV. APPLICATIONS OF THE ULTRACENTRIFUGE TO BIOLOGY AND BIOCHEMISTRY

Centrifuges developing gravitational fields of the order of 5000 to 1,000,000 times gravity are designated as "ultracentrifuges" (978) to distinguish them from the low speed devices used in ordinary laboratory procedures. The latter operate at about 2000 to 4000 r.p.m. and develop fields of the order of 500 to 2500 $\times$ $g$.

Applications of the ultracentrifuge to biological problems have included: (1) the estimation of particle sizes, as in the determination of the molecular weights of proteins; (2) the purification of biological materials, as in the preparation of purified viruses; and (3) the study of the physical properties of protoplasm and the effects of gravitational fields on living forms.

The early development of the ultracentrifuge and much of its subsequent application to biological problems is to be credited to Svedberg and his co-workers, who began to use centrifugal fields about twenty years ago (979) for studying particle size by the rate-of-sedimentation method. They were interested in particles too small (less than about 100 m$\mu$ in diameter) to sediment at appreciable rates under the influence of gravity alone. They soon extended the method to the determination of the molecular weights of protein molecules, values for hemoglobin having been published in 1926 (980).

### 1. Methods

*Types of ultracentrifuges*

Svedberg and his associates have been particularly active in developing oil-driven ultracentrifuges. Details of their machines and of the theoretical principles involved will be found in the book by Svedberg and Pedersen (981). Recent reviews by Svedberg (982, 983) give summaries of essential details and extensive references to the literature. The development of the air-driven centrifuge, following the early work of Henriot and Huguenard (984) is chiefly due to Beams and his associates in this country. For general discussions of Beams' developments, together with citations of original papers, one is referred to two excellent reviews by him (985, 986).

The various types of ultracentrifuges may be classed according to (1) the method of drive, and (2) the method of suspending the rotor. A tabular summary of such centrifuge types and of their characteristics is given in Table XI. The upper limit of fields developed is imposed by the stresses which the rotors can withstand without exploding.

*Determination of molecular weights and particle sizes*

Machines used for this purpose are frequently called "analytical centrifuges." The determination of molecular weights and particle sizes by sedimentation methods is fundamentally a problem in sliding friction, in which two forces—that due to the gravitational field (which is independent of the velocity of the particle) and that due to friction (which is directly proportional to the velocity)—are opposing each other. In its simplest aspects, the solution to the problem is given by Stokes' law, according to which particles free to fall in a suspending medium ultimately attain a constant velocity "$v$" (when the gravitational and frictional forces equal each other) which is related to the particle radius "$r$," the viscosity of the suspending medium "$\eta$," the densities of the particle and of the medium "$dn$" and "$dm$," and the force of gravity "$g$" according to the following expression:

$$r = (9\eta v/[2(dn - dm)g])^{\frac{1}{2}}.$$

For very small particles, it is necessary to take into account factors such as thermal diffusion which do not enter into the above expression. Still other variables are introduced by the use of centrifugal fields. The solution consequently becomes somewhat more complicated, and for details one is referred elsewhere (978, 986). The end result is as follows:

Two methods may be used for calculating the molecular weight. The first is based on the attainment of equilibrium conditions, after which the distribution of concentration of the particles "$c$" with distance "$x$" from the center of rotation is related to the molecular weight "$M$," the gas constant "$R$," the absolute temperature "$T$," the partial specific volume of the solute "$V$," the density of the solution "$\rho$," and the angular

velocity "$\omega$" as follows (982):

$$M=\frac{2RT\log(c_2/c_1)}{(1-V\rho)\omega^2(x_2^2-x_1^2)}.$$

The second is based on measurement of the velocity of sedimentation, which if expressed in terms of the velocity in unit field in water at 20°C is called the sedimentation constant "$s$." If "$\eta$" is the viscosity of the solution and "$\rho$" its density, and if "$\eta_0$" and "$\rho_0$" are the same quantities for water at 20°C, the expression for the sedimentation constant is (982):

$$s=\frac{dx/dt}{\omega^2 x}\frac{\eta}{\eta_0}\frac{(1-V\rho_0)}{(1-V\rho)}.$$

In order to obtain the molecular weight from the sedimentation constant, it is necessary to make use of the diffusion constant "$D$," obtained by independent measurements for which micro-methods are available (982). The molecular weight is then given by (982):

$$M=RTs/[D(1-V\rho)].$$

For observation of the relative concentration of the sedimenting material at different distances from the center of rotation, optical methods based on variations of absorption or of index of refraction are used. In observing such materials as proteins by the absorption method, ultraviolet radiation of wave-length range highly absorbed by proteins (*ca.* 2800A) is employed. The cell is

TABLE XI. *Representative types of ultracentrifuges.*

| Type of Drive | Type of Suspension | Atmos. Surrounding Rotor | Approx. Rotor Radius (cm) | r.p.s. | Field Developed (×g) | References |
|---|---|---|---|---|---|---|
| | | *A. Cell type* | | | | |
| Electric motor | Mechanical (ball bearings) | $H_2$ (760 mm) | 6.5 | 44–280 | 500–20,000 | 981, 983 |
| | Air film | Vacuum ($10^{-5}$–$10^{-7}$ mm) | 5.0 | 1000 | 200,000 | 989(a) |
| | Magnetic | Vacuum ($10^{-5}$–$10^{-7}$ mm) | 7.12 | 1000 | 290,000 | 985, 989(i) |
| Oil turbine | Mechanical (journal bearings) | $H_2$ (20 mm) | 3.25 | 2300 | 700,000 | 989(b) |
| Air turbine | Air film | Air or other gas (760 mm) | 1.40 | 3000 | 450,000 | 986, 989(e) |
| | | Vacuum ($10^{-5}$ mm) | 10.0<br>3.25 | 500<br>2400 | 95,000<br>710,000 | 989(d)<br>989(c and d) |
| Air turbine (driving rotor magnetically) | Magnetic | Vacuum ($10^{-5}$ mm) | 0.74 | 1200 | 430,000 | 989(e) |
| Steam turbine | Air film or steam film | Vacuum | (Similiar to air driven) | | | 989(f) |
| Hydrogen turbine | Air film | Vacuum | 0.45 | 21,800 | 8,250,000 | 989(c) |
| | | *B. Continuous flow type* | | | | |
| Air turbine | Air film | Vacuum | 2.54 (61 cm length)<br>1.90 (9 cm length) (capacity 5 liters per hr.) | 1000<br>500–850 | 100,000<br>19,000–55,000 | 989(g)<br>989(h) |

photographed with such radiation, and a photodensitometer trace is made of the photograph. This permits relative concentration "$c$" to be plotted against distance from the meniscus "$x$." The curves so obtained show steps corresponding to the sedimentation boundaries for the various species of molecules present (Fig. 15).

The variation in index of refraction at the sedimentation boundaries may be used as a means of locating them, either by Toepler's *schlieren* method (988) or by photographing a finely-ruled grating through the sedimentation column (981). In the *schlieren* method, the change in index of refraction at the boundaries results in deflection of the light against a suitably-placed diaphragm which prevents the deflected light from reaching the photographic plate. The boundaries then appear in the photograph as dark lines (Fig. 16). A recent detailed description of the method has been published by Chiles and Severinghaus (989). In the ruled-grating method, the displacement "$z$" of the rulings relative to each other becomes greater, because of changes in refractive index, at the sedimentation boundaries. This relative spacing of the rulings, as determined by photographing the grating through the cell, is plotted against the distance "$x$" from the meniscus. The result is a curve in which the maxima show the positions of the sedimentation boundaries (Fig. 17). A new refractive index method has recently been described by Andersson (1000).

*Purification of biological materials*

The purification of biological materials by ultracentrifugation is based on the difference in sedimentation rates of particles of different sizes. The term "differential centrifuge" is sometimes applied to equipment used for this purpose. In the case of the concentration of large molecules, such as virus proteins, from suspensions containing only very much smaller molecules, the process is essentially an extension of the usual laboratory procedures for removing cells or other particles from suspension. The application of high speed centrifugation to such problems is primarily due to Wyckoff and his associates. The method was first applied to the concentration of pneumococcus antibodies (987), and was soon extended to the crystallization of the tobacco-mosaic-virus protein (988).

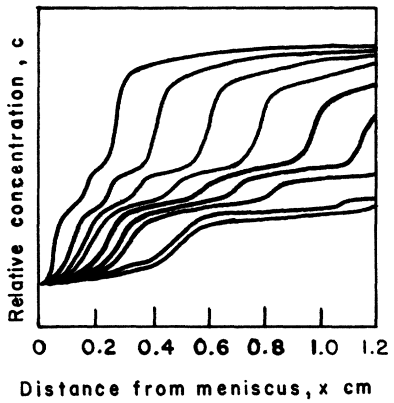



FIG. 15. Examples of sedimentation curves obtained by ultraviolet photographic method. Sedimentation diagram of limulus hemocyanin by Eriksson-Quensel, reference 982, photographed in a field 120,000×g, 5 min. between exposures.

*Effects of centrifugal fields on biological materials*

In order to study the effects of centrifugal fields on biological materials, such as single cells, the materials may be: (a) placed in a container, centrifuged, and then removed for microscopic examination; or (b) observed microscopically while in the centrifugal field, using methods developed by Harvey and Loomis. In Harvey's earliest microscope centrifuge (991), a synchronously-operated mercury vapor light source was used to illuminate the object for a fraction of a second each time it passed beneath the source, thus insuring image steadiness by applying stroboscopic principles. In later designs (992, 993), continuous illumination from a small line filament has been employed. It will be noted (Fig. 18) that the reflections in the optical system change the apparent axis of rotation so that instead of moving around the periphery of a circle the object *appears* to rotate about the center of the microscope field. Under such conditions, if the filament image illuminating the specimen is kept sufficiently narrow, so that the specimen is illuminated during only a small part of its revolution, blurring caused by motion is not objectionable, at least near the center of the field.

Various physical characteristics of cells, such as their surface tension, may be calculated from

their distortion as observed under force fields in the centrifuge microscope. The equations are based upon the application of usual physical principles (994).

## 2. Results

*Molecular weight determinations*

Investigations of the molecular weights of proteins have been the subject of extensive and adequate reviews elsewhere (981–983, 995), and there would be no point in repeating such material here. It will suffice to call attention to the interesting conclusion of Svedberg and his associates that the molecular weights of proteins occur in multiples of a fundamental unit of about 17,600 (see Table XII).

Among recent investigations should be mentioned Kabat's (999) determinations of the molecular weights of antibodies. McFarlane (999a) has compared the sedimentation curves of normal sera with those of sera from various pathological conditions. Interesting differences in the protein components are indicated, as illustrated in Fig. 19. The possibility of using such results as the basis of diagnostic procedures at once suggests itself.

*Purification of biological materials*

Probably the most interesting application of the ultracentrifuge method to the purification of biological materials has been in the preparation of virus proteins. These investigations have been reviewed by Wyckoff (996), Stanley and Loring (997), and by Stanley (998), and need not be discussed in detail here. While other methods (e.g., chemical and ultrafiltration) can be used for the purification of such substances, ultracentrifugation excels by its convenience and efficiency. Results of some of the molecular-weight determinations of purified virus proteins are listed in Table XII.

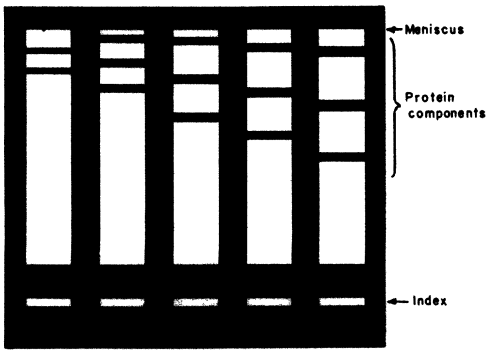



FIG. 16. Type of sedimentation pictures obtained with Toepler's *schlieren* method. From pictures of limulus hemocyanin taken at 5-minute intervals in a field 120,000 $\times g$, by I. B. Eriksson-Quensel, reference 982.

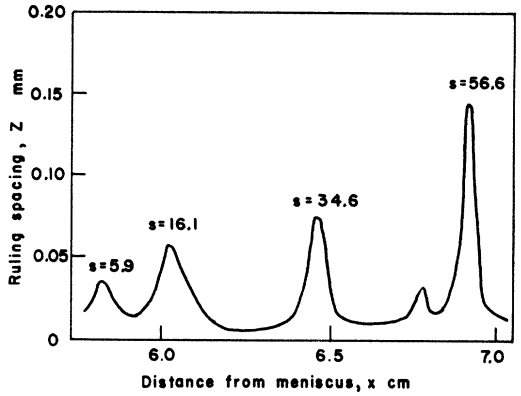



FIG. 17. Example of sedimentation curve obtained with ruled-grating method. Sedimentation diagram for limulus hemocyanin by Pedersen, reference 982, taken 35 minutes after the centrifuge reached full speed, in a field 120,000 $\times g$.

McIntosh and Selbie (1001, 1002) have used the ultracentrifuge for the preparation of purified bacteriophage, as well as virus proteins, and have recently (1003) employed the Sharples continuous centrifuge for preparing such materials in large quantities. In the last work (1003), best results were obtained with *B. coli* bacteriophage. *S. aureus* and *B. prodigiosus* bacteriophages and the Rous chicken sarcoma virus were also concentrated.

*Effects of centrifugal force fields on biological materials*

The materials which have been studied are for the most part single cells, such as *Ascaris* eggs. They may be suspended in a medium of approximately the same density to prevent their rapid motion through the medium under the influence of the field. The effect of the field is to cause a stratification in which the heavier parts of the cell are thrown toward the periphery of the centrifuge (994). As observed under the microscope, the relative densities of different cell structures then become evident at once. Thus, many cells contain oil droplets lighter than the rest of the cell fluid, and yolk granules heavier

TABLE XII. *Selected summary of molecular weight determinations with the ultracentrifuge.*

| SUBSTANCE | MOLECULAR WEIGHT | | MULTIPLE OF 17,600 |
|---|---|---|---|
| | SEDIMENTATION METHOD | EQUILIBRIUM METHOD | |
| *I. Proteins (reference 981)* | | | |
| Erythrocruorin (Lampetra) | 17,100 | 19,100 | 17,600 = 1×17,600 |
| Lactalbumin | 17,400 | | |
| Cytochrome c | 15,600 | | |
| Myoglobin | 16,900 | 17,500 | |
| Gliadin | 27,500 | 27,000 | |
| Hordein | 27,500 | | |
| Erythrocruorin (Arca) | | 33,500 | 35,200 = 2×17,600 |
| Lactoglobulin | 41,500 | 38,000 | |
| Pepsin | 35,500 | 39,000 | |
| Insulin | 41,000 | 35,000 | |
| Ovalbumin | 44,000 | 40,500 | |
| Human tubercle bacillus protein | 32,000 | | |
| Hemoglobin (horse) | 68,000 | 68,000 | 70,400 = 4×17,600 |
| Hemoglobin (man) | 63,000 | | |
| Serum albumin (horse) | 70,000 | 68,000 | |
| Yellow enzyme | 82,000 | 78,000 | |
| Canavalin | 113,000 | | 105,600 = 6×17,600 |
| Serum globulin (horse) | 167,000 | 150,000 | 140,800 = 8×17,600 |
| Serum blobulin (man) | 176,000 | | |
| Antipneumococcus serum globulin (man) | 195,000 | | |
| Edestin | 310,000 | | 282,000 = 16×17,600 |
| Excelsin | 295,000 | | |
| Catalase | 250,000 | | |
| Serum globulin (Lampetra) | 360,000 | | |
| Urease | 480,000 | | 422,000 = 24×17,600 |
| Thyroglobulin (pig) | 650,000 | | |
| Hemocyanin (Homarus) | 760,000 | 800,000 | 845,000 = 48×17,600 |
| Antipneumococcus serum globulin (horse) | 920,000 | | |
| Erythrocruorin (Planorbis) | 1,630,000 | 1,540,000 | 1,690,000 = 96×17,600 |
| Hemocyanin (Octopus) | 2,800,000 | | |
| Hemocyanin (Rossia) | 3,300,000 | | 3,380,000 = 192×17,600 |
| Erythrocruorin (Lumbricus) | 3,150,000 | 2,950,000 | |
| Hemocyanin (Helix pomata) | 6,600,000 | 6,700,000 | 6,760,000 = 384×17,600 |
| Bushy stunt virus | 7,600,000 | | (For other viruses, see *III*, below) |

than the cell fluids, and these collect at opposite extremities of the cells.

Centrifugation in fields of the order of 150,000× *g* does not prevent the subsequent cleavage of uncleaved *Ascaris* eggs, but it does arrest cleavage while they are under the influence of the field (1004, 1005). The ability of living cells to withstand enormous centrifugal fields is especially illustrated by the studies of Beams, King *et al.* (1009) in which centrifuged tissue cultures subjected to fields of 400,000× *g* for half-hour periods continued to grow and to pulsate* after centrifugation.

Harvey (994, 1006) has used the distortion produced in cells by the opposing motions of their yolk granules and naturally-contained or injected oil droplets to measure surface forces in cells. Typical results are: for amoeba, 1–3 dynes per

* Cardiac explants were used.

TABLE XII.—*Continued.*

| | MOLECULAR WEIGHT | DEGREE OF POLYMERIZATION |
|---|---|---|
| *II. Carbohydrates (reference 981)* | | |
| Cellulose acetate in acetone | 50,000–250,000 | 190– 950 |
| Following in cupra-ammonium:<br>Purified cotton linters<br>Most mildly treated cellulose<br>Regenerated cellulose | Cellulose-copper complex:<br>200,000–300,000<br>570,000<br>90,000–110,000 | <br>890–1300<br>3500<br>400– 490 |
| Nitrocellulose in acetone | 102,000–160,000 | 390– 600 |
| Ethyl cellulose in dioxane | 125,000 | 540 |
| *III. Viruses (reference 997)* | | |
| Tobacco mosaic<br>Shope papilloma<br>Equine encephalomyelitis<br>Vaccinia | 43,000,000–52,000,000<br>25,000,000<br>23,000,000<br>2,300,000,000 | (For bushy-stunt virus, see *I*, above) |

cm; for rabbit macrophages, 2; for frog leucocytes, 1.3; and for a slime mold, 0.45.

Guyer and Claus (1007) found that subjection of embryonic and cancer tissues to fields of 400,000× *g* failed to prevent their subsequent growth when implanted in young rats. An interesting observation was that the cancer cells did not stratify appreciably while the embryonic cells did. Luyet and Grell (1008) have used the ultracentrifuge to study the mechanism of death in frozen cells.

These examples indicate the general types of investigations which have been undertaken. Further literature citations will be found in the papers referred to.

### 3. Conclusions

The value of the ultracentrifuge method for measuring the molecular weights of large molecules, such as the proteins, is so well established that it is regarded as a standard technique for such determinations. Use of the ultracentrifuge for the purification of such materials as viruses is also of unquestioned utility. The study of the behavior of cells under high force fields has yielded interesting information, but it is difficult to predict at the moment how much further such investigations can be carried with results that are of fundamental value. The living cell is complex, and the significance of results obtained from the study of it is not always as immediately apparent as is that of data from simple substances.

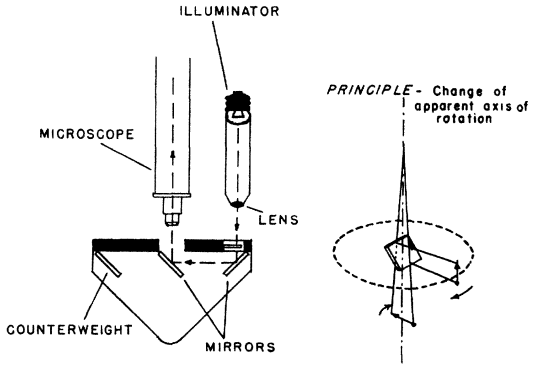



FIG. 18. Professor Harvey's centrifuge microscope, reference 992.

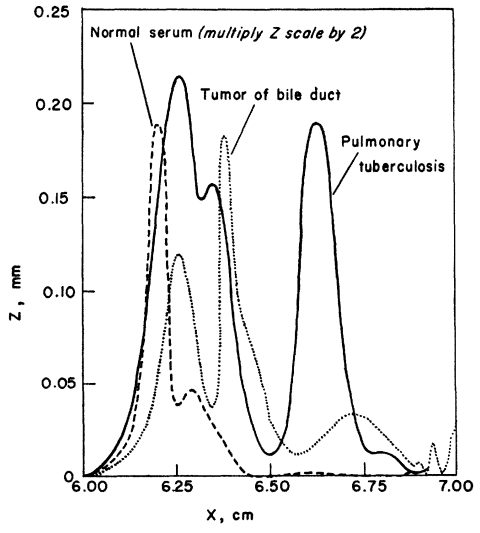



FIG. 19. Changes in the sedimentation curves of human serum in pathologic conditions. From data of McFarlane, reference 999a.

## V. RECENT DEVELOPMENTS IN MICROSCOPY

Of the more recent developments in microscopy applicable to biological problems, ultraviolet and electron microscopy have been chosen for particular discussion as being probably of greatest current interest.

### 1. Ultraviolet microscopy

*Methods*

The apparatus and techniques of ultraviolet microscopy do not differ from those of visible microscopy in fundamental principles, but differences in details are imposed by the types of light sources and optics required, and by the necessity of photographing the field instead of observing it visually. It is possible to use the 3650A mercury line with glass optics, but for shorter wave-lengths quartz must be employed.

The pioneer in ultraviolet microscopy was Köhler (1011). His microscope used quartz lenses corrected for 2750A and not achromatized for other wave-lengths. The usual ultraviolet microscopes of today are based on his designs. They must be employed with monochromatic radiation, and must be separately focused for each wave-length used. Glass objectives designed for 3650A can be achromatized for a wave-length in the visible region and $\lambda = 3650$A, so that focusing may be accomplished visually and the field photographed at the same adjustment by 3650A radiation (1010). In using quartz optics, it is possible to determine experimentally the difference in focusing adjustment required for a particular wave-length in the visible and for each of the wave-lengths to be employed in the ultraviolet. The microscope may then be focused for the visible in each instance and subsequently racked down the predetermined amount to bring it into focus for the ultraviolet wave-length to be used.

As light sources, a mercury arc or cadmium spark may be employed. For 3650A investigations with achromatized glass objectives, filters used in conjunction with a mercury arc give sufficiently monochromatic radiation. For work at shorter wave-lengths, a monochromator is customarily employed to isolate the spectral range desired.

By measuring the transmittancy of the object at different wave-lengths in the ultraviolet, using a photo-cell or other method of determining intensities, ultraviolet absorption spectra of single cells or of parts thereof may be obtained (878, 1012, 1013). This technique has been highly developed by Caspersson and his associates.

The small depth of focus in the ultraviolet may be employed to advantage for "optical sectioning," whereby the size and shape of cell structures is determined by taking series of photographs at different depths within the cell. The method has been described in detail by Lucas (1014).

A comparison of the theoretical resolving powers obtainable at different wave-lengths in the visible and ultraviolet and that of a typical electron microscope is shown in Table XIII. Aside from its slight advantage in resolving power, the quartz ultraviolet microscope has the great advantage over the 3650A glass instrument and the visible microscope that radiation used with it (usually in the range near $\lambda = 2750$A) is highly absorbed by certain cellular structures, particularly those of the nucleus, so that the detailed structure of the cell appears in the photomicrograph without the necessity of staining the preparation. Thus one may observe the structure of live cells. This and the ability to determine, by absorption measurements at different $\lambda$'s, the distribution of such substances as nucleic acids within the cell are the two outstanding features of ultraviolet microscopy.

*Results and conclusions*

The principal contributions of ultraviolet microscopy to biology have thus far been: (1) the study of chromosome structure during various stages of mitosis, with wave-lengths near 2600A highly absorbed by the nucleic-acid-like material of the chromosomes (see Fig. 20); (2) the determination of the ultraviolet absorption spectra of cells and of parts of cells; and (3) the demonstration by such methods of the formation of large amounts of nucleic-acid-like material in the cell at the time of cell division.† The researches of

† Previously referred to in the discussion of absorption spectrophotometry.

TABLE XIII. *Resolving power, μ, of microscopes.*

| λ | RESOLVING POWER, μ N.A. =1.3 | N.A. =1.4 | APPROXIMATE USEFUL MAGNIFICATION* |
|---|---|---|---|
| 5500A | 0.22 | 0.20 | 1750 |
| 4500 | 0.17 | 0.16 | 2100 |
| 3650 | 0.14 | 0.13 | 2600 |
| 2750 | 0.11 | 0.10 | 3500 |
| 2250 | 0.085 | 0.08 | 4200 |
| Electron | 0.005 | | 70,000 |

* Based on a limit of visual acuity of approximately 1 minute of arc.

Caspersson and associates in this field are especially noteworthy (878, 887, 1012, 1015–1017). Among other publications of interest are those of Wyckoff *et al.* (1018–1020), Barnard *et al.* (1021, 1028), Vlés and Gex (1022), Duboulz (1023), Luyet *et al.* (1024, 1025), Lucas *et al.* (1014, 1026), Trivelli (1027), Martin (1029), Schmitt (1030) and Menke (1031). Kazeef (1032, 1171) describes interesting studies with ultraviolet cinematography.

The utility of the method in following nucleic-acid metabolism in cells is well established. It would seem particularly interesting to employ it for following changes in nucleic-acid-like materials within the cell under various physical conditions in investigations of the type discussed in a following section on "intercellular physiology."

## 2. Electron microscopy

*Methods*

In the usual electron microscope, electrons from a hot filament are accelerated in a vacuum through a potential difference of 30,000 to 100,000 volts (corresponding to $\lambda = 0.071$ to 0.039A) against the object under investigation, and magnetic or electric fields are used, in a manner analogous to the employment of lenses for light microscopes, to curve the electron beam after it passes through the specimen and bring it to focus in an enlarged shadowgram image of the object (Fig. 21).

The development of the electron microscope is largely due to Borries, Ruska and their associates (1033–1035). One of their recent papers gives an excellent description of their instrument as produced by Siemens and Halske (1033). Three magnetic lenses (Fig. 22) are used in this and the RCA (1036, 1037) electron microscope; one, corresponding to the microscope condenser, to focus the electron beam on the specimen; one, corresponding to the microscope objective, forming an enlarged, projected image of the specimen; and a third, analogous to the microscope eyepiece, forming a second, further enlarged image. The final image is made visible by a fluorescent screen, or is photographed. With instruments of this type, the resolving power is of the order of 0.005μ, and magnifications of 25,000 to 100,000 times may be obtained. Focusing is accomplished by changing the effective power of the "lenses" through control of the strengths of the magnetic fields.

The specimen must be penetrable by the electron beam for use in the type of electron microscope just described. Biologic specimens may be mounted on thin sheets of Cellophane supported by a metal grid or wire screen. The bombardment by the electron beam subjects them to consider-

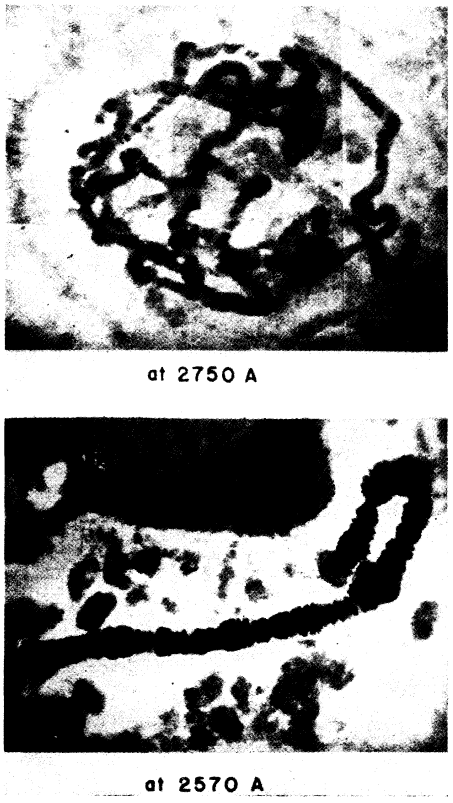



FIG. 20. Ultraviolet photomicrographs of chromosome structure, by Caspersson, reference 878.

able heat. Since electron microscopes operate at high vacuum, time is saved in pumping if the objects are introduced into the previously-evacuated microscope, or removed therefrom, through an air lock of small size. The vacuum, of course, dehydrates the object—a point which must be considered in connection with biological materials. Provisions are usually made for manipulating specimens by fine screws operated through vacuum-tight controls.

In the electron shadow microscope, a shadowgram of the specimen is obtained from a point electron source without the use of lenses (1038–1040). Resolving power of about 0.05$\mu$ is claimed.

Various aspects of electron-microscope design and operation have been discussed by Ardenne (1041), Marton (1042), and others (1043). Books by Meyers (1044) and Ardenne (1045) contain useful information.

Secondary electrons emitted from the object have been used in electron microscopy to form enlarged patterns indicating the positions of such

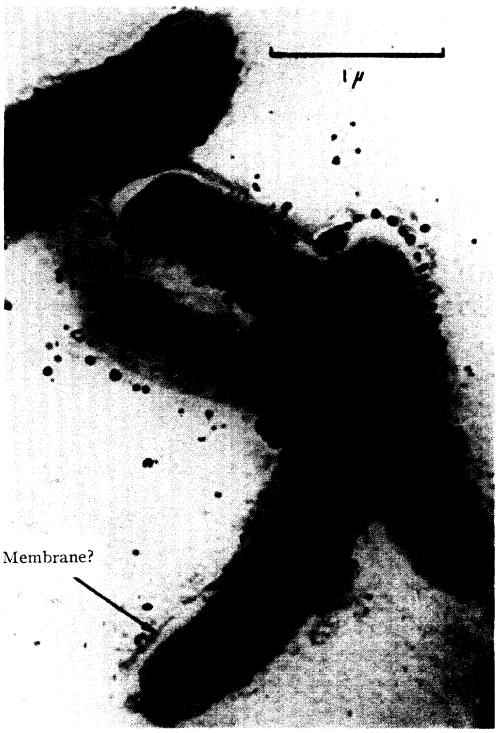



FIG. 21. Photomicrograph of human tubercle bacilli made with RCA electron microscope (40,000×) (Courtesy Dr. V. K. Zworykin).

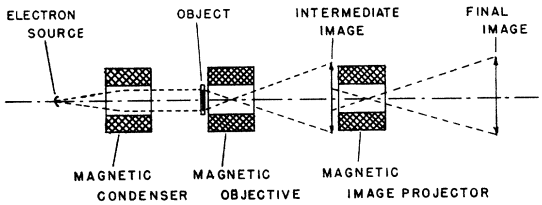



FIG. 22. Schematic diagram of magnetic type of electron microscope.

elements as Ca and Mg in tissue sections (1030, 1047). X-ray microscopes embodying the principle of focusing secondary x-rays from the object in an enlarged image have also been described (1046).

*Results and conclusions*

Many electron-microscope studies of diatoms, viruses, bacteria, etc., have been published (1048–1059). While greater magnifications are obtained than with ordinary microscopes, and while the resolving power is higher, the results are perhaps disappointing as far as indicating new structural details are concerned. Probably improvements in the techniques of handling specimens and of interpreting their photographs will lead, in the future, to more informative findings.

One application of indirect biological interest should be mentioned—the study of the characteristics of dust and smoke particles, including metallic dusts (1060). Quite definite information is obtained in these studies regarding particle shapes and sizes, and these data have important bearing on problems in industrial hygiene.

### 3. Miscellaneous

Fluorescence microscopy is becoming of increasing importance in biologic studies. The method consists in exciting the specimen with long wave ultraviolet (*ca.* 3500A) and observing the visible fluorescent light emitted by it. The principles are essentially the same as in fluorescence spectroscopy (q.v.) except that a microscope is used to examine the object. A suitable ultraviolet source must be used, and filters must be employed between the source and the specimen to eliminate the visible radiation insofar as possible, while at the same time transmitting the exciting ultraviolet. Several reviews of methods

and results have been published (1061–1064). The marked fluorescence of many biologically-interesting substances (e.g., vitamin A) makes their location in cells and tissues possible by this method. Space limitation prevents further discussion of this field, and one is referred to the extensive literature for further information (*viz.*, 946, 947, 1061–1064, 1065).

Interesting infra-red microscopic fields, observed indirectly by means of an electron image tube, have been published by Zworykin and Marton (1066). Finally, attention should be directed to the microscope of Graton and Dane (1067) in which massive mechanical structures are used to improve the effective resolving power by reducing vibrations of the image.

## *VI. OTHER TECHNIQUES*

### 1. Methods of use in cell-physiology research

Until the last ten or fifteen years, the emphasis in physiology had been on the study of the activities of organisms as a whole. Since then attention has been directed more and more to the physiology of the individual cells of which organisms are composed. The change is analogous to that which has taken place in physical research. In the nineteenth century, macroscopic and microscopic problems occupied the preponderance of the physicist's time. In the twentieth century, he turned to submicroscopic investigations, and most of his efforts are now directed toward unravelling the mysteries of molecules, atoms, and subatomic units.

The new era in physical research awaited the solution of numerous problems in the macroscopic realm, and the development of new techniques (x-ray diffraction methods, cyclotrons, etc.) for attacking submicroscopic problems. As with physics, so with physiology. Only since such gross activities of the organism as the circulation of the blood, respiration, hormonal control of body functions, etc. have been comparatively well understood has attention been directed principally to the relation of these grosser activities to those of individual cells, and progress in the latter field has awaited the development of suitable techniques—such as tissue culture and manometric methods. A few of these newer techniques will be discussed, with particular reference to their more physical aspects.

*Tissue culture*

The method consists essentially in the transplantation, under aseptic conditions, of living cells from an animal (1068–1071) or plant (1072) organism into a sterile culture medium, and the subsequent cultivation of such cells or tissues in suitable culture chambers (hollow slides, flasks, etc). It was originally employed successfully by Harrison (1073–1074), and has since been highly developed, especially by Carrel and co-workers, and A. Fischer. Tissue cells of almost any kind—for example: epithelium, muscle, connective tissue, nerve, leucocytes, carcinoma cells, etc.—may be grown in this way, and the method permits their observations free from the complicating influence of the multitude of factors operating in the organism. The cultured cells retain important characteristics which distinguished them before transplantation. Thus, a fragment of embryo chick heart planted in a plasma clot continues to beat as though it were a part of the intact heart, and cultured leucocytes retain their wandering and scavenger-like propensities.

The physical problems involved in tissue culture arise primarily from: (a) the desirability of maintaining constant physicochemical conditions of the fluid and atmosphere within the culture flask, and (b) the need of methods for estimating the growth of cultures.

In the simpler techniques, the cultures are grown in hanging drops on cover slips placed over hollow microscope slides, or in Carrel flasks. Under such circumstances, the culture medium and the atmosphere undergo continual change, because of the metabolic activities of the cells. Nutrient material and oxygen are consumed, and these are replaced by carbon dioxide and other waste products. For many investigations, it is desirable to maintain more constant conditions. Two principal methods have been employed for achieving this end: (1) the use of large quantities of medium in proportion to the amount of tissue,

and (2) the employment of continuous-flow devices in which both the fluid medium and the atmosphere undergo constant replacement. In using the former method, it is necessary that the conditions be such that the large fluid volume will not interfere with the respiration of the cells. This has been achieved by rocking flasks so as to cause the fluid to pass alternately from one side reservoir over the central culture into another reservoir and back again (1075), or by rotating tubes lined with the culture, partially filled with liquid medium, and inclined almost horizontally so that only a portion of the culture is covered by medium at any one time (1076–1079).

The simplest continuous flow method is that of Burrows (1080), in which a cotton wick is used to carry the fluid medium by gravity from a reservoir past the culture to a receiver at a lower level. This did not, however, replenish the atmosphere. A constant-flow device has been described by de Haan (1081), and Carrel has devised a double flask connected to an external piston pump (1075) in which continuous flow of fluid is maintained and the atmosphere is constantly changed. A somewhat simpler arrangement was developed by Lindbergh (1071), in which incoming gas operates an aspirator type of flow device, and the external pump is dispensed with. The problem of designing practical continuous flow devices is not as simple as would appear at first sight, since the method must not be complicated, it must avoid damage to the delicate tissues, it should preferably permit microscopic observation of the tissue during the course of growth, and it should maintain constant composition of both the gas and liquid phases. An interesting extension of present methods might be to connect tissue flasks in tandem, whereby the fluids circulated past a culture subjected to various controlled conditions (such as ultraviolet irradiation) could subsequently be brought in contact with another culture. The effects of substances produced by the first culture and released into the intercellular fluid could then be studied. Lindbergh has developed continuous-flow perfusion equipment by means of which whole organs may be cultured (1082–1085).

For the measurement of the growth of cultures, the most common method is to determine the surface area (enlarged by projection, etc.) at various stages of growth. It is well recognized that this ignores such factors as the thickness of the culture, closeness of packing of the cells, etc., which are important if it is desired to determine the total amount of protoplasm elaborated. One method of overcoming these difficulties is to obtain the dry weights of the cultures with a microbalance (1086–1087). It is difficult, however, to separate the cells from the medium, and after weighing it is not possible to examine the subsequent growth of the particular fragment used. Many attempts have been made to utilize measurements of respiration and glycolysis* as indications of tissue culture growth, but these have not been very successful because of changes in the metabolic characteristics of the tissues during growth (1086, 1089–1095). Optical transmittancy measurements have also been tried, with indifferent success. It is possible that improvements in the optical method (e.g., by the use of monochromatic ultraviolet in the region near 2600A, highly absorbed by nuclear material) might lead to a practicable criterion of growth.

*Manometric methods*

The processes by which living cells obtain their energy are primarily (a) respiration, the oxidative breakdown of carbohydrates to the end products $CO_2$ and water, and (b) glycolysis, the nonoxidative splitting of carbohydrates principally to the end product lactic acid in the case of animal tissue cells (in yeast, the corresponding process is fermentation, in which alcohol, $CO_2$, lactic acid, pyruvic acid, etc. are formed). If glycolysis takes place in the presence of oxygen it is termed "aerobic," if in a *milieu* free from oxygen it is termed "anaerobic." Many types of cells which obtain their energy by glycolysis in the absence of oxygen will change over to an oxidative type of metabolism (respiration) if they are allowed access to oxygen. The presence of oxygen in these cases "suppresses" glycolysis. This is known as the Pasteur effect," because it was first described by Pasteur in the case of suppression of yeast fermentation by oxygen.

Great interest was aroused in cellular respiration, glycolysis and the Pasteur effect by the

* See below for a discussion of these techniques.

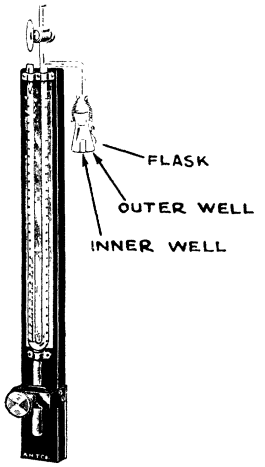



FIG. 23. Barcroft-Warburg manometer.

work of Warburg *et al.*,** which indicated that tumor tissue generally differs from normal tissue in that its ratio of glycolysis to respiration is abnormally high, even in the presence of adequate oxygen supply. The Pasteur effect evidently fails to operate for such tissues. This has led to the generally accepted notion that tumor tissue is characterized by an abnormal carbohydrate metabolism; a clue regarded by many workers as affording the most likely possibility of solving the cancer problem. To be sure, this point of view has been seriously challenged (1099–1101); yet it may be said to be held by the majority of investigators in the field.

Measurements of cellular respiration and glycolysis can be carried out conveniently by micromanometric methods. The same type of equipment may be used for the study of chemical reactions involving small amounts of gas exchange. The manometric techniques used for cell metabolism measurements have been discussed in detail by Dixon (1102) and Fenn (1103).

To illustrate the methods we may consider the measurement of tissue respiration with the Warburg type of manometer (Fig. 23). A small quantity of tissue (e.g., epithelium) is placed in the outer well of the flask in, for example, Ringer's solution, the *p*H being carefully adjusted. The inner well contains a solution of KOH. The indicating fluid in the manometer is Brodie's solution, chosen because its specific gravity is such as to make the calculations convenient. The manometer is placed on a shaking rack, with the flask dipping into a water bath, held at closely constant temperature. The purpose of shaking is to insure diffusion of gases into and out of the fluid in the outer wall. After an equilibrium period to permit all parts of the apparatus to reach constant temperature, etc., the stopcock is closed, the fluid in both sides of the manometer is adjusted to some designated point on the scale (usually 150 mm), and the respiration period begins.

As the tissue respires, it utilizes oxygen and evolves $CO_2$. The latter reacts with the KOH in the inner well. The result is a diminished pressure within the flask side of the manometer system, which results in a rise of fluid in that arm. At the ends of predetermined intervals (usually 15–30 min.), shaking is stopped, the screw controlling the amount of Brodie solution in the manometer tubes is adjusted until the level in the flask side of the manometer is again 150, and the difference in height of the two columns ("$h$") is noted. Since the readings are made at constant volume, they indicate pressure changes. They may be translated into terms of oxygen consumption by means of the following equation, in which "$x$" is the amount of oxygen absorbed in cu. mm at NTP, "$h$" is the difference in height of the manometric columns in mm, "$v_g$" is the volume of gas space in the flask and connecting manometer, "$v_l$" is the volume of liquid in the vessel, "$T$" is the absolute temperature of the water bath, "$P_0$" is $760(13.60/D)$ where "$D$" is the density of the manometer fluid at partial pressure "$P_0$," and "$\alpha$" is the solubility of oxygen at partial pressure "$P_0$" in cu. mm at NTP per cu. mm of fluid.

$$x=(h/P_0)[273v_g/T+v_l\alpha].$$

The Warburg type of manometer is open to the atmosphere, and consequently will record barometric changes. To correct for this, it is customary to use one or more manometers as "barometric controls," and to take for "$h$" the difference "$h_0-h_b$," where "$h_0$" and "$h_b$" correspond to the experimental and barometric manometer readings, respectively (with due regard to proper sign for "$h_b$").

To overcome the variations of readings due to atmospheric pressure changes, the "differential"

** For reviews of Warburg's work and subsequent investigations see references 1096–1098.

manometer may be employed. In this, two flasks are used, forming a closed system. The Barcroft type of differential manometer is similar to the Warburg manometer except for the extra flask closing the system (1102). The Fenn manometer (1103) employs a fine capillary, connecting the flasks, in which a small drop of fluid serves as an index of pressure changes.

To measure glycolysis instead of respiration, the KOH is omitted from the inner well, and bicarbonate solution is added to the outer well. Evolution of gas, due to the neutralization of the lactic acid present, ensues, and this is measured and the glycolysis expressed in terms of it. The abbreviation "$Q$" is customarily employed for the quantity in cu. mm of gas evolved or absorbed or of lactic acid produced *per mg tissue weight.* Subscript "$O_2$" indicates oxygen consumption (thus $Q_{O_2}$ is the quantity of oxygen consumed at NTP); subscript "$m$" or "$l$" indicates lactic-acid production, subscript "$CO_2$" carbon dioxide production, etc. Superscripts indicate the atmosphere present. Thus; $Q_m^{N_2}$ represents quantity of lactic acid produced per mg tissue weight (in terms of cu. mm of $CO_2$ determined experimentally and reduced to NTP) in an atmosphere of $N_2$ (anaerobic glycolysis); $Q_m^{O_2}$ represents the same for an atmosphere of $O_2$ (aerobic glycolysis).

The physical problems involved in manometric techniques are those of eliminating effects of temperature and atmospheric pressure changes, and of improving the accuracies obtainable. Linderstrøm-Lang (1104) has applied the Cartesian diver principle to the construction of very sensitive manometers which he claims can measure quantities of absorbed or evolved gas as small as 0.002 cu. mm. The respiring tissue is placed in the diver. When readings are to be made, the diver is brought to a predetermined mark on the vessel by the application of a known external pressure. Modifications of the Cartesian-diver method have been described by Needham *et al.* (1105). Many other respirometers in addition to the standard Warburg, Barcroft and Fenn types have been described in the literature (1106–1123). Several respirometers have also been devised for measuring the $O_2$ consumption of small animals (1124–1133).

The great need at the present time seems to be for the development of instruments which are automatic and continuously recording, and which are at the same time sufficiently simple and accurate for practical use.

*Methods of use in intercellular-physiology studies*

Despite the principal emphasized so long ago by Claude Bernard that the intercellular *milieu* is as much a part of the organism as the cells themselves, and that the two must be considered together as a functioning whole, and despite the fact that Brown-Séquard and D' Arsonval (1134) pointed out the chemical relationships between cells* long before the hormone hypothesis was postulated, little attention has been paid to the production of materials by cells, which, acting through the intercellular fluids, can control the metabolic activities of neighboring cells. It seems reasonable that community life among cells, devoid as it is of the central nervous control which permits whole organs and tissues to function in harmony, should be regulated by chemical factors produced by the cells themselves. Yet it is only in the plant physiology field—in the study of the bios problem and of growth hormones—that this point of view has been even modestly developed. The animal physiologist seems almost always to turn directly from the study of an organ to the study of the individual cell, so that intracellular rather than intercellular problems occupy his attention.

Certain new techniques for studying intercellular physiology employ physical agents. In 1937, Fardon *et al.*, while studying the effects of ultraviolet radiation on the respiration of yeast (1135), noted that a critical total energy of unfiltered radiation stimulated respiration, while greater or less energy had no stimulative effect. From this, he concluded that over-irradiation killed so many cells that the total respiration was reduced instead of increased, while under-irradiation simply had negligible biologic action. He tried adding ultraviolet-killed cells to a respiring suspension, and obtained stimulation. Eventu-

* "Nous admettons que chaque tissu et plus généralement chaque cellule de l'organisme sécrète pour son propre compte des produits ou des ferments spéciaux qui sont versés dans le sang et qui viennons influencer par l'intermédiare de ce liquide toutes les autres cellules rendues ainsi solidaires les unes des autres, par un mécanisme autre que le système nerveux."

ally, he found that adding *cell-free filtrates* from ultraviolet irradiated cells to a living suspension stimulated respiration.

These results suggested that ultraviolet-irradiated cells produce biochemical factors which stimulate respiration, and encouraged your reviewer and his colleagues to go forward with a series of investigations, still in progress, in which were studied the effects of various physical agents in causing cells to produce and to release into the intercellular fluids factors controlling cellular metabolism. Research has so far been directed primarily toward the investigation of proliferation-promoting and growth-stimulating factors produced by cells in response to various injurious agents—such as lethal ultraviolet light (1136–1151), heat (1152), mechanical vibration (1153), x-rays (1139), etc. They indicate that such factors are produced by the living, intact cells as a physiologic response to injury (1145, 1150, 1152). The individual cells evidently react to injury in this way as a part of the mechanism which results in insuring survival of the cell community under adverse conditions. Some little attention has been paid to the production of respiration, fermentation, and glycolysis-affecting factors under such conditions (1156). By similar methods, Harker (1157) showed that $\gamma$-ray irradiation of yeast leads to an increase in the quantity of sucrose-inverting factors in the intercellular fluid.

The techniques used are always essentially the same in principle. The cells are alive. They are placed in an environment as normal as possible and subjected to the effects of the physical agent. The intercellular fluids are then obtained, free from cells, by centrifugation, filtration, etc., and (either before or after concentrating them) their effect on other (usually homologous) cells is tested. The use of physical agents instead of chemical agents is to be preferred in such studies because it permits the careful control of conditions and obviates the difficulty, always present when chemicals are used, of carrying the original agent over in the cell-free fluids.

Only a beginning has been made in the application of such techniques, and one can visualize many possibilities for further study. For example, while a preliminary inquiry has been made into the influence of lack of oxygen on the production by cells of proliferation-promoting (1155), and respiration and glycolysis stimulating factors (1156), no thorough investigation has been made. This question naturally occurs: When cells are subjected to oxygen lack, will they produce factors stimulating the glycolytic processes, or factors stimulating respiration? It seems unlikely that they would exhibit no response at all.

There is no reason of course, other than the difficulties mentioned above, why chemical agents cannot also be employed. A start has been made with chemical agents in the investigation of the influence of $\beta$-indole-acetic acid (1154) and of carcinogenic hydrocarbons by this technique. To the physicist, however, the many possibilities in the use of physical agents seem the most intriguing.

### 2. Miscellaneous methods

*Photography and cinematography*

The usual methods and applications of photography to macro- and micro-experimental investigations will not be considered, as they are treated in detail in numerous texts.

Interesting applications of infra-red photography to paleobotanical research have been described by Walton (1158). Several papers have appeared on methods of applying cinematography to bacteriological research (1159–1164). Some of the most intriguing microcinematographic studies have been those in which the growth and multiplication of cells in tissue cultures have been photographed (1165–1171). By the use of interval timers, permitting the taking of exposures at intervals of considerable length, the "action" can be speeded up so that the processes of cell division, growth, etc., appear to take place rapidly before one's eyes. The method is of use in the studying of differences in growth in different kinds of normal tissues, and in pathologic as contrasted with normal tissues. Many biological applications of photomicrography, with both visible and ultraviolet light, are described in papers by Kazeef (1032, 1171).

*Electrocardiography and electroencephalography*

The use of the electrocardiograph in the diagnosis of pathologic conditions of the heart is so well known that descriptions of instruments and their applications will be found in almost any standard textbook on human physiology. Atten-

tion may be directed, however, to the important technical improvements in the method which have been made possible by the substitution of the cathode-ray oscillograph for the older string galvanometer as a means of indicating action currents. In the last ten years, considerable interest has been aroused in the action currents produced in the brain. These may be observed by connecting electrodes, applied to the scalp, to suitable amplifying and recording equipment. The resulting record, called an "electroencephalogram" shows marked deviations from the normal in the case of certain mental disturbances (1172), particularly epilepsy (1173), and such records are said to be of use in the localization of brain tumors (1174, 1175).

*Fragmentation by supersonic vibrations*

Ultrasonic vibrations have been used for the mechanical depolymerization of starch and gelatin (1176, 1177), and for the fragmentation of the protein hemocyanin from *Helix pomata* (1178). In the latter case, submolecular units one-half and one-eight the size of the original molecule were obtained. Chambers and Flosdorf (1179) have also employed such vibrations for the fragmentation of bacteria in the extraction of labile constituents from them. They point out that certain heat-labile proteins are stable to sonic waves (1180), and that the method should therefore make possible the extraction of numerous materials not obtainable from heat-killed or formalin-killed bacterial suspensions. A highly labile antibody-combining constituent was extracted from *S. haemolyticus* in this way.

*Photoelectric determination of microorganism population density*

Photoelectric densitometers, or nephelometers, are coming into increasing use for estimating microorganism populations in bacteriological investigations (1190–1196). These devices depend upon measurements of the transmittancy or light scattering power of microorganism suspensions. Such determinations can be translated into terms of cell count or of total weight of protoplasm by means of previously determined calibration curves. It has been shown, at least in the case of certain micro-organisms, that the measured transmittancy is a truer indication of total protoplasm than of cell count (1194–1196). Useful electronic circuits applicable to photodensitometric measurements have been described by Shepard (1197).

*Surface film methods*

It would not be fitting to close this section without brief mention of the surface chemistry techniques originated by Lord Rayleigh and Miss Pockels, and subsequently developed by many workers—especially Langmuir, Adam, Rideal and their co-workers. The method consists essentially in forming monomolecular layers on water of such materials as fats and fatty acids, and in studying their properties under various conditions. Among other things, it is possible to deduce the cross sections and lengths of molecules in these monolayers from the area and thickness of the film and the quantity and molecular weight of the substance of which it is composed. Several books and reviews have appeared on the subject (1198–1203), and one is referred to them for details. The properties of protein monolayers have been reviewed recently by Langmuir (1201) and Danielli (1202). In Professor Rideal's laboratory at Cambridge, many fascinating investigations concerning biochemical reactions in surface films, effects of radiation on surface films, etc., have been undertaken. For the results, one should consult the recent literature by Rideal and his associates.

*Mathematical biophysics*

In this field may be grouped numerous inquiries in which the aim has usually been to reach a better understanding of physiologic processes by undertaking to express them mathematically. Often an attempt has been made to deduce from the forms of the resulting equations the kinds of factors involved in the processes.

The mathematical approach has been applied extensively to the analysis of growth curves for individual organisms and populations, and of curves of survival and decay. Such work has been reviewed by Wilson (1181), Davenport (1182), Bernstein (1183), Winsor (1184) and Lotka (1185), among others. Rashevsky has applied mathematical analysis to problems of cell multiplication (1186), nerve transmission (1187), etc.

(1188, 1189). Wrinch's development of the cyclol hypothesis (discussed above) was largely based on geometrical considerations, and was in this sense a mathematical approach to a biochemical problem.

There are, of course, endless possibilities of combining mathematical analyses with experimental investigations. In particular, statistical methods are frequently employed as criteria of the validity of experimental results. The problems usually classed as "mathematical biophysics" are, however, in a different category. They are approached from the purely analytical point of view, using the experimental data of other investigators.

The experimentalist using mathematics to analyze his data can go back to the laboratory at any time to check matters which are brought into question or to obtain new information which turns out to be needed in order to clarify the problem. The strictly mathematical biophysicist is limited by the data provided for him. The utility of his investigations depends upon whether they actually lead to simpler or more precise ways of stating or explaining the physiological problems dealt with, and the ultimate criterion of the validity of his mathematical deductions should be laboratory tests by competent experimentalists.

In physical research the two methods of approach—theoretical and experimental—have been satisfactorily developed by specialists in each without sacrifice of progress. Whether an analogous specialization will prove equally fruitful in dealing with the less precise data of biology remains to be seen.

## *VII. ACKNOWLEDGMENTS, PART I*

Even in the extended space so kindly put at the disposal of your reviewer by the editors of this journal, it has been impossible to discuss all useful methods in detail. In instances, therefore, in which reference to important investigations have been omitted, the indulgence of those concerned is requested.

A review seems more useful if an endeavor is made to evaluate the work rather than simply to report what has been done. This philosophy has been adopted in preparing the preceding material. It is subject to the danger that comments and opinions may be colored by the reviewer's personal bias, but every effort has been made to avoid this.

It would be impossible to prepare a review of this extent without help from many kind persons. Thanks are due Professor S. C. Brooks, Professor I. L. Chaikoff, Professor G. v. Hevesy, Dr. W. T. Astbury, Professor E. K. Rideal, Dr. Albert Fischer, my former colleague Dr. E. S. Cook, my colleague Professor I. W. Sizer and many others for their kindness in reading and criticizing portions of the manuscript or in extending the hospitality of their laboratories. Especial thanks are due my very able assistant, Miss Lois Joyce, for her unremitting care in checking questionable points, and Miss Helen Ann Beyersdoerfer for her painstaking efforts in preparing the typescript from longhand copy which no one else could decipher.

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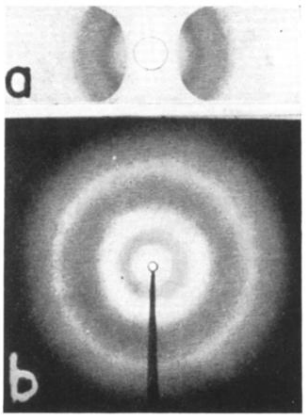


FIG. 1. (a) X-ray diffraction pattern of water, after Warren, reference 431. (b) Powder-type pattern of heat-denatured hemoglobin, after Clark, reference 407.

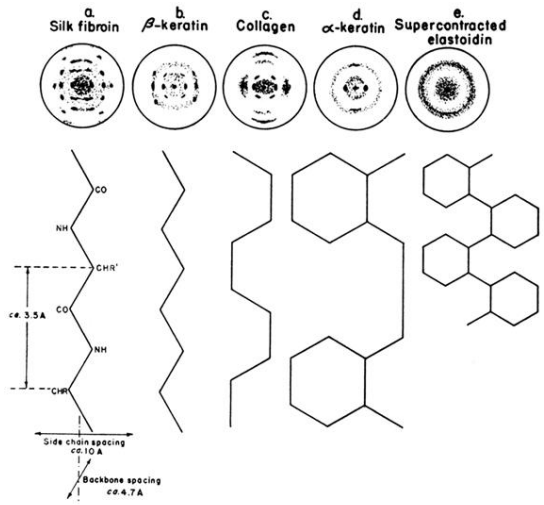


FIG. 2. Idealized x-ray fiber patterns of various protein fibers and their interpretation in terms of the configuration of the protein molecules.

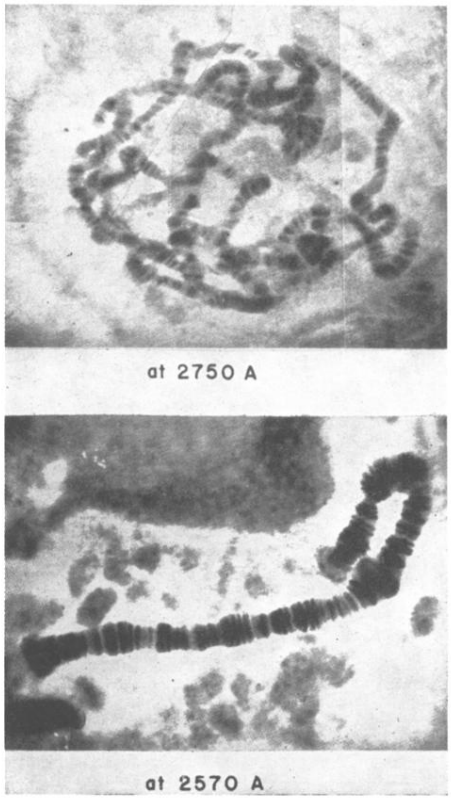


FIG. 20. Ultraviolet photomicrographs of chromosome structure, by Caspersson, reference 878.

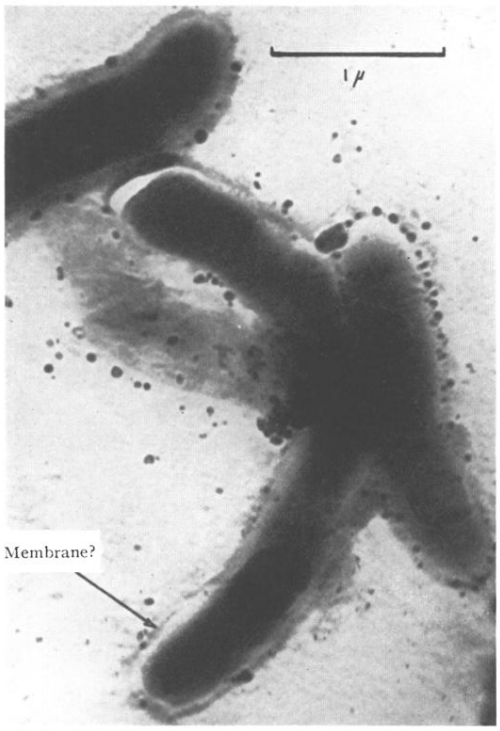


FIG. 21. Photomicrograph of human tubercle bacilli made with RCA electron microscope (40,000×) (Courtesy Dr. V. K. Zworykin).

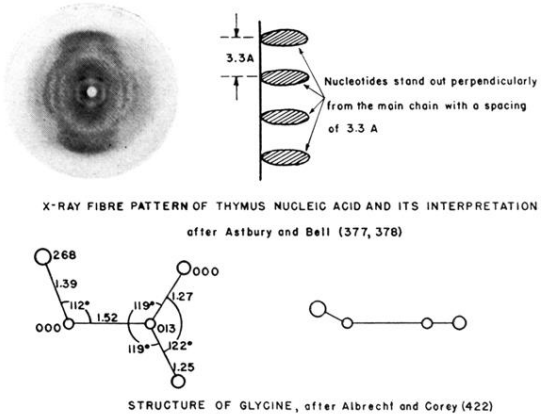


FIG. 4. X-ray diffraction studies of thymus nucleic acid and glycine.