Antibiotics as Tools for Metabolic Studies. III. Effects of Oligomycin and Aurovertin on the Swelling and Contraction Processes of Mitochondria*

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The activities of oligomycin and aurovertin, both known to inhibit oxidative phosphorylation, have been compared with respect to their effects on the mitochondrial swelling and contracting processes. Oligomycin retards the participation of ATP in the processes of (a) protection of mitochondria against orthophosphate-induced swelling, (b) contraction of thyroxine-swollen mitochondria, and (c) induction of increased extent of swelling at *p*H 6.1. Aurovertin displays slight or no reversal of these activities. Aurovertin decreases the time of onset of swelling induced by thyroxine; apart from this, neither antibiotic shows a marked effect on swelling in the absence of ATP. Magnesium is able to depress the effectiveness of both antibiotics. Both oligomycin and aurovertin act synergistically with oxidizable substrate to prevent swelling. The results of these experiments are discussed with reference to the relationship between swelling and respiratory-phosphorylative mechanisms.

The systematic application of enzyme or enzyme-complex inhibitors to the study of metabolic processes has repeatedly demonstrated the value of this approach. The finding that the antibiotics oligomycin (Lardy *et al.*, 1958) and, more recently, aurovertin (Lardy *et al.*, 1964) are potent inhibitors of the oxidative-phosphorylation machinery has provided additional means for the elucidation of the mechanism of phosphate esterification and the associated reactions. Indeed, the effects of these antibiotics have been observed in phosphorylation in the exchange reactions between $^{32}P_i$ and ATP, in the exchange of ^{18}O between P_i and H_2O and in ATPase activity (Lardy *et al.*, 1958, 1964; Lardy and McMurray, 1959). Although these two antibiotics display nearly identical inhibitory effects on phosphorylating or coupled systems (including exchange reactions), of especial import is the finding that they exert quite different effects on mitochondrial ATPase activities, at least one type of which is generally regarded as a reverse of the phosphorylation mechanism (Lardy and Wellman, 1953).

The many proposals (Hunter *et al.*, 1959; Lehninger, 1959; Packer, 1961) that the process of mitochondrial swelling is intimately involved with phosphorylation and respiratory intermediates suggest that an investigation of the effects of oligomycin and aurovertin on swelling might contribute to our knowledge of the chemistry of oxidative phosphorylation as well as to a better understanding of the swelling phenomenon. Furthermore, such a study would provide an additional approach to a comparison of inhibitory activities of oligomycin and aurovertin.

This paper describes the results of experiments designed to test and compare the effects of oligomycin and aurovertin on swelling induced by phosphate, ATP, or thyroxine, and on protection against and reversal of swelling by ATP.

EXPERIMENTAL

Mitochondria were prepared from rat liver according to the procedure described by Lardy and Wellman (1952) except that the final volume of the particulate suspension was equal in ml to the grams of tissue used. The mitochondria were stored in 0.25 M sucrose at 0°. In agreement with the work of Tapley (1956), Hunter *et al.* (1959), and Emmelot (1962), it was observed that subtle but definite alterations in swelling response occurred after aging at 0° for as little as 2 hours. In view of this every effort was made to run the experiments as soon as practicable after preparation of mitochondria.

The swelling and reversal experiments were carried out in matched 15 × 150–mm tubes. The time, rate, and amount of optical density change was followed using an Evelyn colorimeter with a special tube adapter and a 515-mμ filter. Timing limitations allowed the simultaneous running of up to six tubes. In all cases the experiment was begun by adding mitochondria to the medium. The composition of the medium varied and will be described in the legends. Mitochondria from 150 mg of tissue, in 0.15 ml suspension, diluted to 6.0 ml had an optical density at 515 mμ of about 0.65 in a medium of KCl. In experiments involving 0.25 M sucrose, 0.10 ml suspension diluted to 6.0 ml had an optical density at 515 mμ of 0.45. The mixture was rapidly swirled and the initial optical density was read immediately. Additions were made to the incubating mixtures as indicated in the figures. The volume of these additions was kept minimal and the optical density was recorded before and after each. All incubations were conducted at 24°.

Doubly distilled water and highest purity chemicals were used throughout. The antibiotics are described by Lardy *et al.* (1964); the A isomer of oligomycin was used in all work described in this paper.

RESULTS

Because the processes of mitochondrial swelling and contraction cannot yet be described in terms of chemical mechanisms it is necessary to choose enough criteria to provide an adequate description of swelling occurring in any particular environment. Figure 1 presents three criteria which must be considered in evaluating swelling data.

Extent of swelling is defined as the difference between the initial optical density and the optical density of the plateau reached after swelling occurs. Such a level may represent the amount of physiologically controllable membrane "relaxation" which mitochondria are capable of in a given situation. It must be pointed out that often a gradual decrease in optical density occurs

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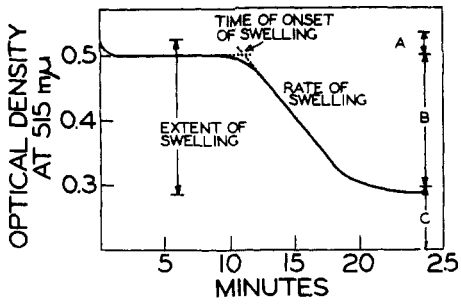

FIG. 1.—Key diagram for swelling terminology. (A) Rapid osmotic change involving primarily intermembrane space; (B) physiologically controllable and reversible alteration of inner membrane; (C) gradual osmotic change involving inner mitochondrial space.

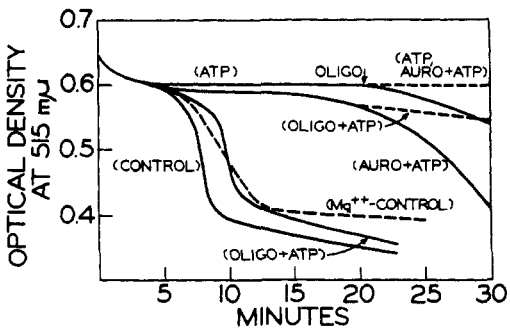

FIG. 2.—Effect of oligomycin A and aurovertin on the antiswelling influence of ATP. The medium is 0.112 M KCl; 0.017 M Tris-HCl, *p*H 7.4; 0.005 M phosphate; 0.15 ml mitochondrial suspension (see Methods) and, when added, 0.001 M $MgCl_2$; 0.0033 M ATP, and 4 μg antibiotic in a total volume of 6.0 ml. Components present before addition of mitochondria are shown in parentheses. Oligomycin was added to one tube (upper solid line) during the experiment as indicated by the short arrow. Broken lines indicate presence of magnesium.

even after the rapid swelling process. This gradual incorporation of water (Fonnesu and Davies, 1956) may be a result of a physiologically uncontrollable osmotic change in mitochondrial volume. Since a true plateau may not appear in such instances the relative extent of swelling is based on the optical density reached at the longest practicable time.

The phenomenon of mitochondrial swelling as defined by change of optical density may represent a composite of three separate influences: a very rapid osmotic change (A) involving the sucrose-accessible space between the double membrane, a controllable change (B) with reference to time and amount, involving the inner membrane, and finally a more gradual osmotic change (C) involving the sucrose-inaccessible space (Fig. 1) (Malamed and Recknagel, 1959; Tedeschi and Harris, 1955; Lehninger *et al.*, 1959; Lehninger, 1960; Avi-Dor, 1960).

Time of onset (*TO*) is defined as the time when the swelling process, as observed by change in optical density, begins. This time is estimated by extrapolation as shown in Figure 1.

Rate of swelling is defined as the slope of the optical density-time plot between the T_0 and the lower plateau. This criterion has shown no consistent relationship to the extent or T_0 but since it does vary considerably under different circumstances its inclusion as an important descriptive criterion is warranted, at least for the present. These factors will be referred to, as defined here, in this and subsequent reports concerning swelling (Connelly and Lardy, 1964).

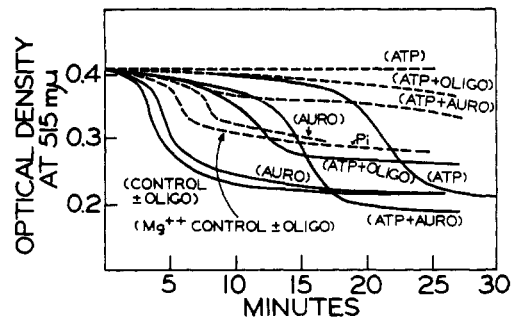

FIG. 3.—Effect of oligomycin A and aurovertin on the antiswelling influence of ATP in sucrose media. The medium is 0.14 M sucrose; 0.017 M Tris-HCl, *p*H 7.4; 0.005 M phosphate; 0.10 ml mitochondria suspension, and, when added, 0.001 M $MgCl_2$; 0.0016 M ATP and 4 μg antibiotics. The addition of P_i at the arrow brought the concentration to 10^{-2} M.

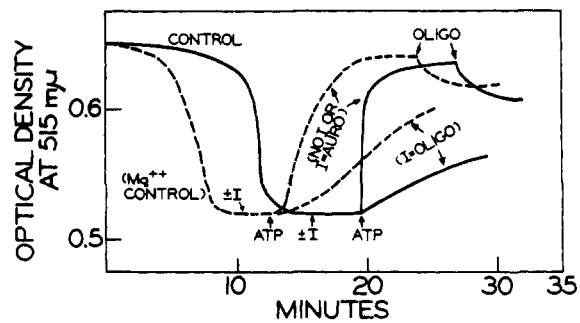

FIG. 4.—Effect of oligomycin A and aurovertin on ATP-induced contraction of thyroxine-swelled mitochondria. The medium is 0.112 M KCl; 0.017 M Tris-HCl, *p*H 7.4; 10^{-4} M thyroxine; 0.15 ml mitochondrial suspension and, when added, 0.001 M $MgCl_2$; 0.0033 M ATP; 4 μg antibiotics.

*Effect of Oligomycin and Aurovertin on Mitochondrial Swelling Induced by Inorganic Phosphate at p*H *7.4.*—The protective effect of ATP against swelling, especially in the presence of certain divalent cations such as Mg^{2+}, has been observed by Raaflaub (1953), Hunter and Ford (1955), Tapley (1956), and Lehninger (1960). If this activity of ATP is related in any way to oxidative phosphorylation (or ATPase activity) the value of ATP as an antiswelling agent might be diminished by chemicals known to inhibit the oxidative phosphorylation process. Such is the case with oligomycin, which, although displaying little or no capacity to swell or to contract mitochondria, acts to depress the effects of ATP (Fig. 2 and 3) (see Neubert and Lehninger, 1962).

On the other hand aurovertin, also a potent inhibitor of oxidative phosphorylation and oxygen-18 exchange, but not of certain ATPase activities (Lardy, 1961; Lardy *et al.*, 1964), has relatively little influence on the effects of ATP on swelling. The difference in the effects of these two antibiotics on protective activity of ATP at *p*H 7.4 is more marked in KCl media than in sucrose (Fig. 2 and 3). As shown in Figure 2, oligomycin prevents ATP from increasing T_0 as compared with the control. When oligomycin is added to a system already containing ATP, a decrease in optical density occurs immediately, but more gradually than when the mitochondria were treated with the antibiotic before the addition of ATP. The effects of aurovertin are similar to those of oligomycin, but the former acts more slowly or with less potency.

Magnesium, which tends to decrease the extent of swelling (Baltscheffsky, 1957) and, more important, to

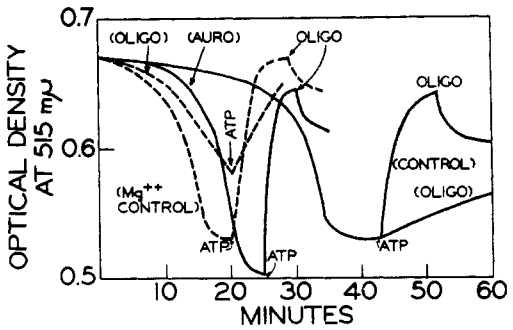

FIG. 5.—Susceptibility of mitochondria treated with oligomycin A or aurovertin to the contractile influence of ATP. The medium is the same as in Fig. 4. The course of swelling with oligomycin present was identical with that of the control until the time of adding ATP.

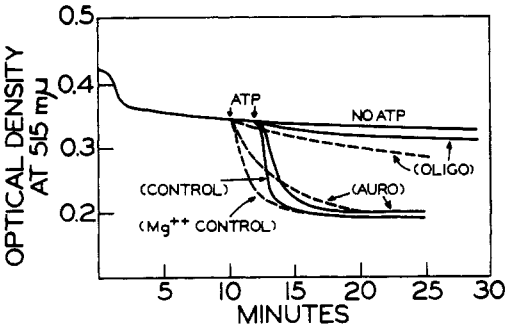

FIG. 6.—Susceptibility of oligomycin- and aurovertin-treated mitochondria to the swelling activity of ATP at *p*H 6.1. The medium is the same as in Fig. 3 except that 0.017 M histidine-HCl, *p*H 6.1, replaces Tris buffer.

enhance the effects of ATP, counters the action of both antibiotics. Mitochondrial swelling in the presence of aurovertin plus Mg^{2+} and ATP is not different from that in the presence of ATP or ATP plus Mg^{2+}. The effect of oligomycin is still apparent but obviously repressed by the combination of ATP plus Mg^{2+}. No attempt to investigate the relationship of inhibitor and Mg^{2+} ion concentrations was made. Occasionally, in these experiments, ATP was added after T_o and in these instances swelling was never prevented although the rate was greatly decreased. Furthermore, a few attempts to contract swollen mitochondria with ATP alone were unsuccessful.

In sucrose media (Fig. 3) the effects of oligomycin, in the concentrations employed here, are essentially the same as observed in KCl but less pronounced. Such a variation could conceivably be a consequence of the inhibitory action of sucrose (Johnson and Lardy, 1958; Lehninger, 1959; Chappell and Greville, 1961). Nevertheless, the inhibition by oligomycin of ATP protection and the effect of Mg^{2+} on this inhibition is consistent with the results observed with KCl media. The potency of aurovertin as an antagonist of ATP protection seems to be increased in sucrose.

Experimental observations of the tendency of Mg^{2+} to decrease extent and increase T_o (Raaflaub, 1953) are further substantiated by the data shown in Figure 3. The addition of either antibiotic to a phosphate plus Mg^{2+} ion system has little effect on Mg^{2+}-induced changes, and excess phosphate will not act to increase extent.

Effect of Oligomycin and Aurovertin on Thyroxine-induced Swelling and ATP-induced Contraction.—The influences of oligomycin and aurovertin on thyroxine-induced swelling and subsequent contraction were compared. Mitochondria, in a KCl medium, were allowed to swell to completion under the influence of thyroxine. The antibiotic was added (except for the control) and within a few minutes a quantity of ATP sufficient for contraction (Lehninger, 1959) was added. Figure 4 indicates the ineffectiveness of aurovertin as compared with oligomycin in blocking the effect of ATP. In addition to limiting the extent of contraction to about 50% of the control, oligomycin at the level 4 μg/6 ml markedly decreases the rate of contraction. Even when added to ATP-contracted mitochondria, oligomycin reverses the contractile process (Figs. 4 and 5). Nearly identical effects of the antibiotics are observed when Mg^{2+} is present in the medium. In contrast to the effects of Mg^{2+} ions in phosphate-induced swelling, Mg^{2+} noticeably lowers the T_o of thyroxine-induced swelling, does not vary the extent, and lowers the rate of contraction slightly. Consistent with previous observations is the antagonistic effect of Mg^{2+} upon oligomycin. Although contraction by ATP with Mg^{2+} is greatly impeded by oligomycin it appears that extensive contraction is possible. The fact that addition of oligomycin to the contracted mitochondria results in a partial reversal of contraction, even in the presence of Mg^{2+} ion, suggests that when the influence of ATP is blocked by oligomycin, thyroxine continues to act as a swelling agent.

The effects of the two antibiotics on thyroxine-induced swelling vary slightly in different experiments. That oligomycin has no effect on T_o or extent is clearly shown in the solid curve of Figure 5. There is no difference between the two conditions *until ATP is added.* Aurovertin, however, decreased the T_o and slightly increased extent. In some experiments oligomycin seemed to decrease the rate of swelling somewhat, similar to the variation in rate between the two magnesium-containing systems (Fig. 5). Likewise, in some experiments aurovertin displayed less tendency to vary from the control than that shown in Figure 5. The results of these experiments in regard to contraction are identical to those of Figure 4.

Effect of Oligomycin and Aurovertin on ATP-induced Swelling at pH 6.1.—Under certain circumstances, namely, in the presence of phosphate at *p*H 6.1, ATP allows a marked and generally prompt increase in extent of mitochondrial swelling over that observed in the absence of ATP (Connelly and Lardy, 1964). The effects of oligomycin and aurovertin, both in the presence and absence of Mg^{2+} ions, are shown in Figure 6. Oligomycin acts to prevent or at least drastically reduce the rate of swelling induced by ATP. This inhibitor action is also observed in the presence of magnesium. Aurovertin shows only a very slight tendency to inhibit the effectiveness of ATP. Aurovertin is slightly more effective in the presence of magnesium.

Figure 7 shows results obtained when ATP is present from the beginning. Under these circumstances the rate of swelling is greatly decreased as compared with that induced by later addition of ATP (see also Connelly and Lardy, 1964). Although oligomycin or aurovertin shows little effect on the phosphate-induced decrease in optical density (Fig. 6), in the presence of Mg^{2+} ions these antibiotics assist in slowing this initial swelling rate. Finally, aurovertin, in the presence of magnesium, appears to be more potent in opposing the action of ATP than when ATP is added subsequent to the antibiotic (Fig. 6), although the qualitative aspects are similar.

Effect of Oligomycin, Aurovertin, and Substrate on Phosphate-induced Mitochondrial Swelling at pH 7.4.—Although the data of Chappell and Greville (1958) show that addition of substrate to phosphate-treated

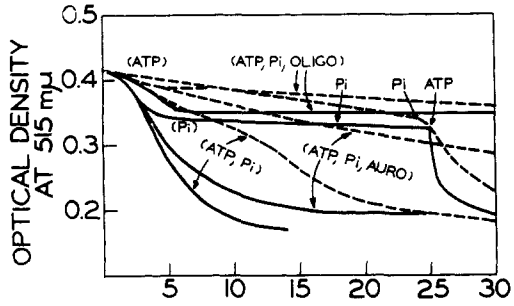

FIG. 7.—Effect of oligomycin A and aurovertin on ATP swelling activity at *p*H 6.1. The medium is the same as in Fig. 6.

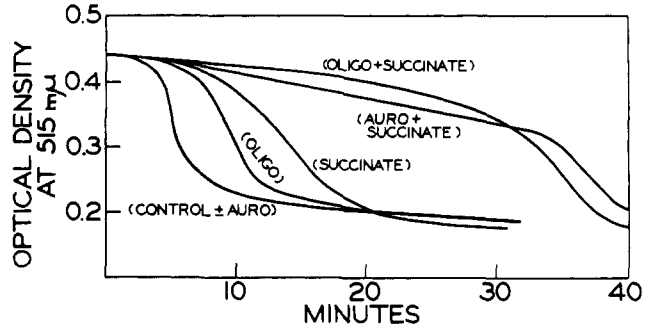

FIG. 8.—Effect of oligomycin A and aurovertin on antiswelling influence of succinate. The medium is 0.14 M sucrose; 0.017 M Tris-HCl, *p*H 7.4; 0.005 M phosphate; 0.10 ml mitochondrial suspension and, when added, 0.0033 M succinate, 4 μg oligomycin, and 10 μg aurovertin.

mitochondria produces a decrease in T_0, we have consistently observed a slight protective effect at *p*H 7.4 by moderate amounts (*ca.* 3 mM) of either succinate or glutamate.

The variance in such findings is conceivably owing to a number of differences in experimental conditions, and the types of response elicited by substrate may be dependent on the status of the mitochondria employed. Nevertheless, the synergistic protective effect offered by substrate plus either antibiotic is worthy of note (Fig. 8). Oligomycin alone shows only slight tendency to increase T_0; aurovertin shows none. In combination with succinate, however, the swelling process is noticeably deferred to about the same degree with both antibiotics and without any significant variation in extent. DNP 10^{-5} M prevents a postponement of T_0 by substrate plus antibiotic. Furthermore, when DNP is added during the protective phase an immediate increase in rate ensues until swelling is complete.

At *p*H 6.1 the addition of substrate, especially succinate, to phosphate-treated mitochondria results in an increase in extent similar in degree to that observed when ATP is added to a phosphate system. Both oligomycin and aurovertin are without effect in this case and swelling continues in the presence of either antibiotic at an unvaried rate. These observations are given more complete consideration by Connelly and Lardy (1964).

Discussion

Within the past decade research has provided considerable evidence for the interdependence of respiration, phosphorylation, and membrane integrity mechanisms (Lehninger, 1962). The existence of an intermediate, or intermediates, common to these functions has been suggested to explain various experimental observations (Chance, 1963). The following facts, relevant to the location of such intermediates, emerge from the present and previous studies.

The site of oligomycin inhibition is separate from the reactions of the respiratory chain and from that portion of the energy-transducing machinery which remains functional in systems uncoupled by DNP (Lardy *et al.*, 1958; Lardy and McMurray, 1959; Slater, 1963). This is illustrated by the fact that, although oligomycin inhibits ATP-$^{32}P_i$ and P_i-$H_2{}^{18}O$ exchanges, 2,4-dinitrophenol and other uncoupling agents are capable of releasing oligomycin-blocked respiration. Aurovertin rigidly parallels these effects of oligomycin (Lardy *et al.*, 1964) as well as its effect on prolonging T_0 in the presence of substrate (Fig. 8).

Oligomycin interferes with the utilization of ATP by mitochondrial ATPase and the membrane phenomena. Examples of the latter are (a) the antiswelling activity of ATP, (b) the contraction of swollen mitochondria by ATP, and (c) the induction by ATP of increase in extent of orthophosphate-induced swelling at low *p*H (Connelly and Lardy, 1964). These activities may be attributed to a single oligomycin-sensitive mechanism. The observed effects of aurovertin on these activities is markedly different from those of oligomycin.

If one accepts that ATPase activity and ATP participation in contraction and swelling of mitochondria involve reversal of ATP generation in oxidative phosphorylation reactions, the differences between the two antibiotics studied here may be rationalized. It appears that aurovertin and oligomycin are equally inhibitory to the phosphorylation reactions proceeding in the direction of ATP formation. They differ, however, in their effectiveness as inhibitors of these reactions in the direction of ATP utilization. In the three general types of ATP utilization described in this work, i.e., *p*H 7.4 antiswelling, contraction of thyroxin-swollen mitochondria, and induction of swelling at low *p*H, aurovertin inhibited less than oligomycin and, under some conditions, not at all. These phenomena parallel the effects of aurovertin and oligomycin on ATPases induced by valinomycin, tribromophenol, aging, and thyroid hormones (Lardy, 1961; Lardy *et al.*, 1964).

Schematic diagrams may be formulated to explain the existence of a high-energy intermediate common to oxidative phosphorylation, and ATPase activity, transhydrogenation, or ion transport (see Chance, 1963). Analogous schemes may be formulated to explain the influence of ATP, oxidizable substrate, *p*H, and oligomycin on structural changes in mitochondria (Connelly and Lardy, 1964).

However, the effects of aurovertin on these mitochondrial functions indicate the existence of energy-transfer systems far more complicated than those hitherto proposed. The only scheme we have been able to formulate consistent with the findings involves a high-energy complex (W) formed irreversibly from either ATP or respiratory activity (Lardy *et al.*, 1964). This intermediate would not be on the direct oxidative phosphorylation pathway. Its formation from ATP would be inhibited by oligomycin but not by aurovertin. Its formation via a nonphosphorylated high-energy intermediate of oxidative phosphorylation would not be inhibited by either oligomycin or aurovertin. The hydrolysis of this complex (W) would be the basis of one form of uncoupling of oxidative phosphorylation or ATPase activity. Thyroid hormones (Lardy, 1961), valinomycin (Moore and Pressman, 1964), parathyroid hormone (Fang *et al.*, 1963), and, to a slight extent, DNP and tricyanoaminopropene (Lardy, 1961) participate in the

hydrolysis or utilization of the energy of complex W. The Mg-stimulated ATPase of aged mitochondria presumably is mediated via W. Also W probably participates in ion transport and in mitochondrial swelling and contraction phenomena.

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The Subcellular Site of Hexosamine Incorporation into Liver Protein*

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The incorporation of hexosamine into subcellular components known to be implicated in protein synthesis was investigated by adding [^{14}C]glucosamine to blood perfusing the isolated rat liver *in vitro*. The deoxycholate-soluble (membranous) protein fraction of microsomes proved to be a major site of hexosamine incorporation. Relatively little incorporation of hexosamine into either ribosomal or soluble cytoplasmic protein was observed. The isolation of radioactive glucosamine from glycopeptides derived from the membranous fraction of microsomes provided evidence that at least part of the hexosamine radioactivity was in fact incorporated into polypeptide chains of glycoproteins. These data support the conclusion that hexosamine or hexosamine-containing oligosaccharides are incorporated into completed peptide chains in the membranes of the endoplasmic reticulum, after their release from the ribosomes.

Considerable evidence is currently available to indicate that the ribosomes are the subcellular site of incorporation of amino acids into a growing peptide chain; this is followed by release of the completed polypeptide into the membranous portion of the microsomes (Littlefield and Keller, 1957; Kirsch *et al.*, 1960; Dintzis, 1961). However, relatively little is known about the subcellular sites and mechanisms involved in the completion of the tertiary structure of proteins such as folding, the formation of disulfide bonds, or the attachment of prosthetic groups such as carbohydrates.

Recent evidence that the liver is a primary site for the covalent incorporation of carbohydrates into serum glycoproteins has been obtained in several laboratories (Spiro, 1959; Shetlar, 1961; Sarcione, 1962, 1963; Robinson *et al.*, 1964). In a previous study (Sarcione, 1964), evidence was presented that galactose and mannose are incorporated into peptide chains within the membranous fraction of liver microsomes subsequent to completion of polypeptide synthesis by the ribosomes. Since an aspartyl-glucosamine linkage has been established in several different glycoproteins (Johansen *et al.*, 1961; Eylar, 1962; Izumi *et al.*, 1962), while galactose has been shown to occupy a penultimate position (Eylar and Jeanloz, 1962), it is possible that the subcellular site and mechanisms for the insertion of hexosamine into polypeptide chains may be different from that of hexoses.

In the currently accepted scheme for protein synthesis, a number of alternative pathways for the incorporation of hexosamine into polypeptide chains can be envisaged. For example, hexosamine could be first linked to aspartic acid and the activated complex incorporated into the growing peptide chain on the ribosomal template. Or, hexosamine could be incorporated into completed peptide chains while they remain bound to the ribosomal template. Finally, hexosamine could

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