THE ACTION OF STREPTOMYCIN

III. The Action of Streptomycin in Tissue Homogenates[1]

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The previous papers (Umbreit, 1949; Oginsky *et al.*, 1949) demonstrate that one of the actions of streptomycin is to inhibit the condensation between oxalacetate and pyruvate in susceptible strains of *Escherichia coli*. It is, however, well known that the oxalacetate-pyruvate condensation is an important reaction in the animal cell. Why, then, is streptomycin able to enter the animal body and kill or inhibit the susceptible bacteria therein without harm, or at least comparable harm, to the animal?

There are at least three possible explanations. First, it is possible that the oxalacetate-pyruvate condensation in the animal differs in some way from the same reaction in the bacteria. A very attractive concept is that in the animal the pyruvate is first converted to "active acetate," which is the material condensing with the oxalacetate. Indeed, such a system has recently been reported for animal tissue (Stern and Ochoa, 1949). One might suppose that the bacteria possess a "direct" condensation (Oginsky *et al.*, 1949) of pyruvate and oxalacetate not involving "active acetate." Second, it is possible that the reactions in the animal and the bacteria are essentially identical but that streptomycin does not penetrate to the site of this reaction in the animal cell, whereas it does so in the bacteria. Third, it is possible that streptomycin is itself converted into a toxic substance by the enzymes of the bacterial cells, but that the animal does not possess this enzyme system.

The importance of the problem of why streptomycin is able to enter the animal body without appreciable harm to the animal necessitated study of the effect of streptomycin in animal tissues or preparations therefrom. Although the methods employed are necessarily those of animal metabolic studies, the problem is one of most interest to the bacteriologist and is therefore recorded here. The terms "water homogenate" and "isotonic homogenate" are defined under methods, but it is helpful at this point to mention briefly the recent information on the enzymatic properties of mitochondria. When it was demonstrated (Bensley and Hoerr, 1934; Hoerr, 1943; Claude, 1942, 1944; Claude and Fullam, 1945; Hogeboom, Schneider, and Pallade, 1947, 1948) that it was possible to obtain morphologically intact mitochondrial fractions from animal tissues by the use of isotonic media for the disruption of the cell, the enzymatic properties of these fractions were studied (Hogeboom, Claude, and Hotchkiss, 1946; Schneider, 1946*a*,*b*; Kennedy and Lehninger, 1948; LePage and Schneider, 1948; Schneider, 1948; Schneider, Claude, and Hogeboom, 1948). It has become apparent that

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the "citric acid cycle" component enzymes and carriers are associated with the mitochondria. A more important consideration is that the mitochondria behave differently when carefully prepared in isotonic media than when "roughly" treated, as in water homogenates, and do not require supplementation with diffusible cofactors. The enzymes in or on these carefully prepared particles appear to be more accessible to certain metabolites than they are in the tissue (Cohen and Hayano, 1946), but they possess properties that sometimes differ greatly from the comparable enzymes isolated in the cell-free state. The reason for these differences, which most workers agree do exist, is a matter of present study.

However, to distinguish among the several possible mechanisms of streptomycin action cited above, it is necessary to demonstrate that one of two phenomena occurs: first, that the enzyme causing the reaction in the animal is not susceptible to streptomycin even in the cell-free state (thus showing that the enzyme systems are different, hypothesis 1 or 3), or, secondly, that it is susceptible to streptomycin providing one permits the streptomycin to penetrate to the centers of this reaction in the animal cell (hypothesis 2).

In tissue slices or isotonic homogenates of rat liver or rat kidney very high concentrations of streptomycin, of the order of 200 to 300 μg per ml, frequently have no effect. At times a level of 300 μg per ml will show some inhibition in homogenates. Lower levels of streptomycin show no effect. However, studies on the distribution and rates of excretion of streptomycin in the animal body (Boxer, Edison, and Hawkins, 1949) have led to the conclusion that little if any of the streptomycin penetrates the cell, most of it being, indeed, distributed in the extracellular body fluids. One is therefore inclined to begin with the hypothesis that streptomycin does not penetrate to the enzyme catalyzing the oxalacetate-pyruvate condensation.

Since in both isotonic and water homogenates the cell structure is destroyed and one has a suspension of cell-free particles (nuclei, mitochondria, and submicroscopic particles with few if any intact cells), one might presume that any permeability barrier to streptomycin would be eliminated. However, this is not necessarily the case, and the fact that in order to preserve the mitochondria one must employ isotonic media and that such isotonic preparations do not usually require supplementation with diffusible factors such as DPN or cytochrome c indicates that a "permeability" barrier still exists at the surface of the mitochondria.[2] The problem that concerned us was whether or not this barrier pre-

[2] An alternative hypothesis is currently being developed by Green and co-workers (Green, Loomis, and Auerbach, 1948; Cross, Taggart, Covo, and Green, 1949) under the name "cyclophorase." It is our understanding that the "cyclophorase" concept conceives of the differences between mitochondrial preparations and the individual enzymes after isolation as being due, not to a permeability barrier at the mitochondrial surface, but to the existence of these enzymes in a different state. One conceives of a very large protein aggregate with the enzymes and coenzymes locked into it in some degree of order. Such a mechanism would account for the phenomenon we now speak of as permeability effects. Homogenization in water, for example, could be thought of as either disrupting certain portions of this aggregate or as interfering with the permeability. Agents that alter these phenomena may be spoken of as altering permeability or as, in Green's terminology, "transforming agents." Experimental evidence as to which of these two views is the explanation

vents the streptomycin from reaching the oxalacetate-pyruvate condensation enzyme. This enzyme has been shown to be concentrated in the mitochondria (Schneider and Potter, 1949).

METHODS

Homogenates were made in an all-glass homogenizer, as described by Potter (1945), and the techniques and reagents described by Potter, LePage, and Klug (1948) and Potter, Pardee, and Lyle (1948) for determining oxalacetate oxidation were followed. The components used in the complete system were glass-distilled water to make 3.0 ml final volume, 0.4 ml of 0.5 M KCl, 0.1 ml of 0.1 M $MgCl_2$, 0.1 ml of 0.1 M K phosphate (pH 7.4), 0.1 ml of 0.01 M KATP (or K adenylate), and 0.5 ml of isotonic KCl (if water homogenates were used). If less than 0.5 ml of isotonic homogenates were used, sufficient isotonic KCl was added to be equivalent to 0.5 ml. As oxidation substrates, 0.2 ml of 0.1 M pyruvate, 0.2 ml of 0.1 M oxalacetate, or an equivalent amount of 0.1 M fumarate was employed. The oxalacetate was prepared just before use. ATP was purified from commercial products by the methods described by LePage (1945). There was little difference between supplementation with ATP or adenylate; in most cases the latter was employed. Since it has been shown (Pardee and Potter, 1948) that fumarate will not be oxidized in this system unless the oxalacetate formed is removed, fumarate was frequently substituted for oxalacetate, particularly when long incubation periods were employed.

Two types of homogenates were used. Each was prepared with 9 ml of fluid per gram of fresh tissue. The "water homogenates" were prepared with ice-cold glass-distilled water; the "isotonic homogenates" were prepared with either 1.15 per cent KCl (to which had been added 0.3 ml of 0.1 M $KHCO_3$ per 100 ml to give a pH of 7.5) or with 8.5 per cent sucrose. We found very little difference between 0.9, 1.15, and 1.23 per cent KCl, or 8.5 per cent sucrose; hence for convenience only the data with 1.15 per cent KCl in kidney homogenates is reported. Liver and brain tissues were examined with entirely comparable results. We found little effect following the supplementation of isotonic homogenates further with cytochrome c and DPN; hence such supplements were used only when water homogenates were employed. The amounts used were 0.4 ml of cytochrome c (5 mg per ml) and 0.1 ml K DPN (coenzyme I, diphosphopyridine nucleotide) equivalent to 330 μg of DPN. We are indebted to Dr. G. A. Emerson for the animals, to Dr. J. E. Hawkins for specially treated animals, to Dr. G. A. LePage, University of Wisconsin, for a sample of purified ATP, and to Drs. LePage and V. R. Potter, University of Wisconsin, for very helpful advice. Streptomycin or dihydrostreptomycin was always employed in the form of the hydrochloride.

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for the observed phenomena is not sufficiently developed to render possible a valid decision between them at this time. However, rather than cite the two indistinguishable alternatives we have expressed the explanations and experiments in this paper in terms of the permeability hypothesis, recognizing that the "barrier" we speak of may be a chemical dislocation rather than a distinct membrane.

EXPERIMENTAL RESULTS

The problem of why streptomycin can enter the animal body without marked toxicity to the animal was approached through two types of experiments. The first approach consisted of comparing the activity of streptomycin in isotonic homogenates and in water homogenates, and was based upon the presumption that in the water homogenate the permeability properties of the mitochondria were impaired.

Figure 1 shows data on kidney homogenates comparing the activity on a mixed oxalacetate and pyruvate substrate in the presence or absence of 120 μg per ml of streptomycin. Dotted lines represent streptomycin treatment. To attain comparable conditions both systems carried out the oxidation in isotonic KCl supplemented with cytochrome c and DPN in addition to the usual supplements of ATP and magnesium. The data given are for unwashed homogenates

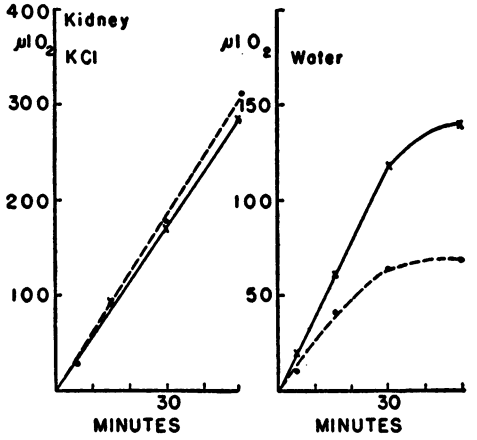

Figure 1. Comparative action of streptomycin on isotonic and water homogenates from rat kidney. Streptomycin (dotted lines) at a level of 120 μg (free base) per ml. Substrate, oxalacetate and pyruvate. Supplemented with cytochrome c and DPN. Thirty mg wet weight of tissue per cup.

used at a level of 30 mg wet weight of tissue per Warburg cup. At this dilution the addition of pyruvate alone results in very little oxygen uptake. In the isotonic KCl homogenates the activity is relatively constant for more than an hour; in the water homogenates it drops off rapidly after 30 to 40 minutes. From these data it is apparent that streptomycin inhibits the reaction in the water homogenates. However, relatively high levels of streptomycin are still required, concentrations in the range of 100 μg per ml being necessary. Concentrations lower than this have no effect.

The second approach to the problem of whether or not there exists a barrier to streptomycin at the surface of the mitochondria consisted of attempts to inhibit the reaction in isotonic homogenates by lowered concentrations of streptomycin under conditions designed to overcome or minimize any permeability barrier. Two types of experiments were employed. Data from the first

type are illustrated in figure 2. A kidney KCl homogenate was added to the cold reaction flasks and oxygen uptake was determined immediately after preparation. The data (curves A, figure 2) show no inhibition at a level of 60 μg of streptomycin per ml. A portion of this homogenate was held for 2 hours in the refrigerator. Oxygen uptake was then determined as before. The data (curves B, figure 2) show no inhibition by streptomycin. A third portion was added to the cold reaction flasks complete with substrate (and in one case streptomycin) and held for 2 hours in the refrigerator; oxygen uptake was then determined (curves C, figure 2). A fourth portion was treated in the same manner except that the substrates were added just before the end of the 2-hour refrigeration period (curves D, figure 2). When streptomycin has been in contact with the mitochondria for a period of 2 hours in the cold, an inhibition is evident. No

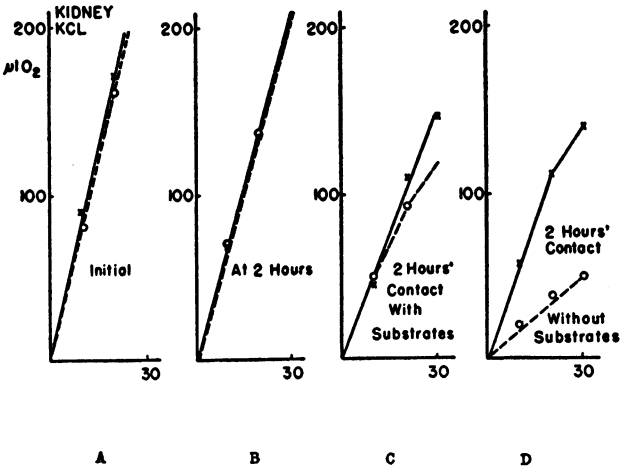

Figure 2. Influence of time of contact on the effect of streptomycin in isotonic rat kidney homogenates. Streptomycin (dotted lines) at a level of 60 μg (free base) per ml. Substrate, fumarate and pyruvate. Thirty mg wet weight tissue per cup. No additions of cytochrome c or DPN. See text for details.

supplementation with cytochrome c or DPN was used. Fumarate, which acts the same as oxalacetate, was employed here in order to circumvent the spontaneous decomposition of oxalacetate during the 2-hour refrigeration period. Two factors, however, have been varied in this experiment. In those cases in which streptomycin showed inhibition, not only was streptomycin present but the homogenate was diluted 1 to 6. This probably does not invalidate the results since the comparable flasks without streptomycin were also diluted to the same extent, and no great loss of activity is evident. However, experiments were designed to alter only one variable, that of streptomycin concentration. An isotonic homogenate was prepared and 0.6 ml (= 60 mg wet weight of tissue) were added to each of 7 cold empty Warburg flasks. Two of these were supplied with the reaction mixture, consisting of buffer, isotonic KCl, Mg^{++}, adenylic acid, oxalacetate, and pyruvate, and used immediately (one flask also received 100 μg strepto-

mycin). The remaining 5 received 0.1 ml of $MgCl_2$ and 0.3 ml isotonic KCl or dilutions of streptomycin in isotonic KCl so that the homogenate (thus diluted to 1.0 ml) would contain 0, 10, 25, 50, and 100 μg streptomycin per ml, respectively. This series was held in the refrigerator for 2 hours, the reaction substrates and supplements (less Mg, volume = 2 ml) were added and the oxygen uptake was measured. In this case the final concentration of streptomycin per flask at the time that oxidation occurred was only one-third that of the streptomycin recorded. Thus the homogenate exposed to 100 μg per ml of streptomycin oxidized the oxalacetate at a concentration of only 33 μg per ml. The concentration of streptomycin to which the mitochondria were exposed, however, is used in plotting the data shown in figure 3. Here it is evident that detectable inhibition is observed at as low at 10 μg per ml, and increasing inhibitions are evident at higher concentrations.

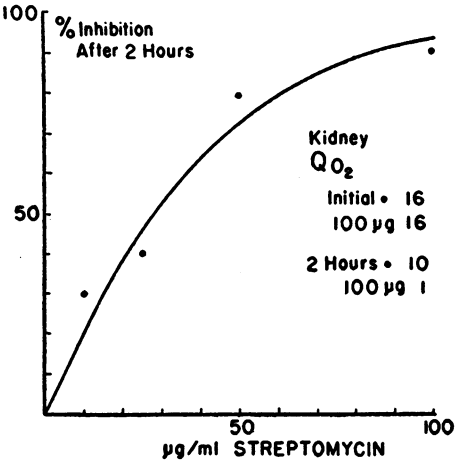

Figure 3. Degree of inhibition after 2-hour exposure to streptomycin. Details in text. Substrate, fumarate and pyruvate. Isotonic KCl homogenates.

DISCUSSION

It seems evident that the reaction carrying out the oxalacetate-pyruvate condensation in the animal *can* be inhibited by streptomycin. In the intact animal and, indeed, in isotonic preparations from the animal the reaction is not inhibited unless and until certain barriers, which resemble permeability barriers, are overcome. We have observed streptomycin inhibition at relatively low concentrations of streptomycin (50 to 60 μg per ml) in isotonic homogenates of liver, kidney, and brain whenever these homogenates were subjected to somewhat drastic conditions. Thus in "cyclophorase" preparations that were not adequately neutralized or in homogenates in which some heating occurred during homogenization, whenever activity remained it was more sensitive to streptomycin than the same activity in adequately treated preparations.

Two further points are of interest. In studies on penetration such as those of

figure 3, dihydrostreptomycin showed much less inhibition than streptomycin. It therefore appeared that it may have "penetrated" more slowly.

It was consequently of interest to determine whether, in the tissue of animals showing the effects of chronic toxicity of streptomycin or in those from animals acutely poisoned by streptomycin, the oxalacetate-pyruvate condensation was in any way impaired. Dr. J. E. Hawkins provided us with several animals of each type. Liver, kidney, and brain tissues of rats showing the vestibular and auditory symptoms of chronic streptomycin toxicity and those from rats that had been killed by intravenous or intercranial injections of streptomycin showed oxidation of oxalacetate-pyruvate at the same rate as controls.

SUMMARY

Streptomycin will inhibit the oxalacetate-pyruvate condensation in animal tissue as well as in the bacterial cell. In the intact animal permeability factors apparently prevent streptomycin from acting on the site of this reaction. These permeability barriers exist not only at the cell wall, but also at the mitochondria, and may be chemical as well as physical in nature. When the barriers are overcome, however, streptomycin does inhibit the oxalacetate-pyruvate condensation in animal tissue.

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