THE ACTION OF STREPTOMYCIN

VI. A New Metabolic Intermediate

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In attempting to explain the results obtained when certain strains of *Escherichia coli* were treated with streptomycin, a reaction between pyruvate and oxalacetate was proposed as the sensitive reaction. The action of streptomycin on this reaction was regarded as being related to its action in inhibiting the organism since only antibiotically active forms of streptomycin were inhibitory and complete inhibition was obtained at concentrations comparable to those required to inhibit growth (Umbreit, 1949; Oginsky *et al.*, 1949). The nature of the "oxalacetate-pyruvate" reaction has remained obscure since further studies (Umbreit *et al.*, 1951) have shown that it was not identical with any of the known reactions of these compounds. Since the conversion of pyruvate to active acetyl and the condensation of this with oxalacetate to form citrate have been studied intensively, and since this reaction series would seem to account for the oxalacetate-pyruvate effect, it was quite unlikely that a new reaction would be found. However, we were unable to demonstrate any effect of streptomycin on the known reactions of pyruvate or oxalacetate (Umbreit *et al.*, 1951; Umbreit, 1952*a*,*b*) even at concentrations of streptomycin far above those required to inhibit growth. To the best of our knowledge no one else has been able to demonstrate inhibition of the known metabolic reactions of these compounds. One paper (Barkulis, 1951) reports streptomycin inhibition of pyruvate metabolism under anaerobic conditions, but the data available do not specify the reaction involved. Since the effect is maximal in the presence of bicarbonate, and since it may be shown that the reactions pyruvate to acetate plus formate and formate to hydrogen plus carbon dioxide are not sensitive to streptomycin *per se*, we have supposed that these studies pointed to a different way to measure the same reaction and were a reflection of streptomycin inhibition of the oxalacetate-pyruvate reaction.

If streptomycin did not inhibit the known reactions, what was the nature of the unknown reaction? However, the experimental problem was not easily approachable because of two very large difficulties. First, it proved most difficult to obtain any kind of preparation which would contain a streptomycin sensitive reaction measurable by the rather indirect methods it was necessary to employ (either the oxidation system of Oginsky *et al.*, 1949, or the anaerobic system of Barkulis, 1951). There were indications that this eventually would be possible since a few active preparations were obtained (Umbreit, 1949), but as an experimental tool this type of approach was, and still is, impossible. Preparations could be obtained which were capable of carrying out the known reactions of pyruvate and oxalacetate, but these reactions were not inhibited by streptomycin. It appeared that the streptomycin sensitive reaction was dependent largely upon the intact cell which would suggest either that the cell was conducting an additional reaction(s) of some quantitative significance or that the action of streptomycin had something to do with the "intactness" of the cell. Second, there was the lack of any way to measure the sensitive reaction except its inhibition by streptomycin. Under these circumstances the only way one could identify the sensitive reaction was to stop it with streptomycin so that a search for end products was fruitless. It seemed probable that one was dealing with an active intermediate, probably present in small amounts, for which one had no method of measurement except to prevent either its formation or metabolism with streptomycin.

Always, however, when streptomycin showed inhibition, the presence of both pyruvate and a four-carbon dicarboxy acid was required; the latter either added or the conditions required were such as to permit its formation. Attention was turned therefore to the possibility that a seven-carbon intermediate played an important

role in metabolism; a possibility that had never quite been eliminated but for which there was no positive evidence. Studies of the oxalocitramalic acid of Martius (1943) or of the dihydro-oxalocitramalic acid as possible intermediates proved negative. Similarly the metabolic properties of several other seven-carbon compounds (including shikimic acid and its relatives)[1] could not be related to streptomycin inhibition. Of course, other seven-carbon intermediates of metabolic significance might exist. We therefore turned our attention to the seven-carbon *phosphorylated* compound, isolated by Rapoport and Wagner (1951) from dog liver, whose structure was indicated as 2-phospho-4-hydroxy-4-carboxy-adipic acid (illustrated in figure 1) which we shall call the new compound in the remainder of this paper. Because of the content of phosphorus in the new compound it was possible to trace its formation. Three problems were especially pertinent. First, how may one isolate and determine this compound? Second, is it an active metabolic intermediate? Third, is it related to the action of streptomycin? It is the purpose of this paper to provide the experimental answers to these problems.

MATERIALS AND METHODS

Chemical methods. Procedures for the isolation and characterization of the compound are outlined in the first section. One point is of considerable importance when dealing with compounds of this structure and requires some modification of technique. The usual method of determining radioactivity of inorganic phosphate in the presence of organic phosphate is to precipitate the former with magnesium. It is assumed generally that very little organic phosphorus is carried down in the precipitate especially during a reprecipitation. However, because the new compound is highly acidic and readily adsorbable, we became suspicious of this procedure. It was found by experiment that while 15 per cent of the phosphorus found in the first magnesium precipitate was organic rather than inorganic when prepared from the original trichloracetic acid extract of liver (11 per cent from ester phosphorus fraction), some 78 per cent of the phosphorus was organic in magnesium precipitates from concentrates of the new compound (85 per cent was organic in magnesium precipitates from nucleic acid fractions). Earlier work might well have discarded a great share of this compound in the removal of inorganic phosphate when magnesium was employed. To circumvent this difficulty we used the method of Ennor and Rosenberg (1952) which consists of extracting the reduced orthophosphate-molybdate complex with isobutanol. The extract separates the inorganic (ortho) phosphate, which then may be read colorimetrically and plated directly for the measurement of radioactivity. However, in some cases we have found extraction of the organic phosphate into the isobutanol layer. In these cases we applied the method of Ennor and Rosenberg to magnesium precipitates of inorganic phosphate, especially in fractions containing the new compound. The remainder of the techniques employed are essentially standard (Umbreit *et al.*, 1949). Radioactivity was determined by use of a thin window Geiger-Müller counter. All samples were counted to an accuracy of ±5 per cent, and the appropriate corrections were made for decay, background, and coincidence. All of the experiments employing P^{32} were done with the active cooperation of Dr. I. Clark, without whose facilities and advice the experiments would not have been possible.

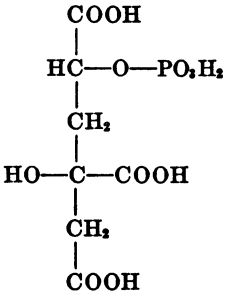

Figure 1. Formula for the new phosphorus compound.

RESULTS

Isolation and determination of the new compound. The compound isolated by Rapoport and Wagner (1951) from dog liver had the formula $C_7H_{11}O_{11}P$ and had properties corresponding to the formula shown in figure 1. This material was discovered originally (Rapoport and Wagner, 1947; Rapo-

[1] We are greatly indebted to Doctors E. F. Rogers and K. Pfister of the Research and Development Laboratories, Merck and Company, Inc., for an array of these compounds.

port and Nelson, 1945) as a nitrogen-free acid resistant phosphorus contaminant of adenosine triphosphate prepared by the usual barium and mercury precipitation methods prevalent at the time. These properties provided a clue for a method of isolation which is used as a method of materials into substances whose barium salts are soluble. The mercury is removed and the new compound precipitated, essentially pure, with barium acetate (pH 8.2). The inorganic phosphate was separated for counting by the isobutanol extraction procedure described, and the total

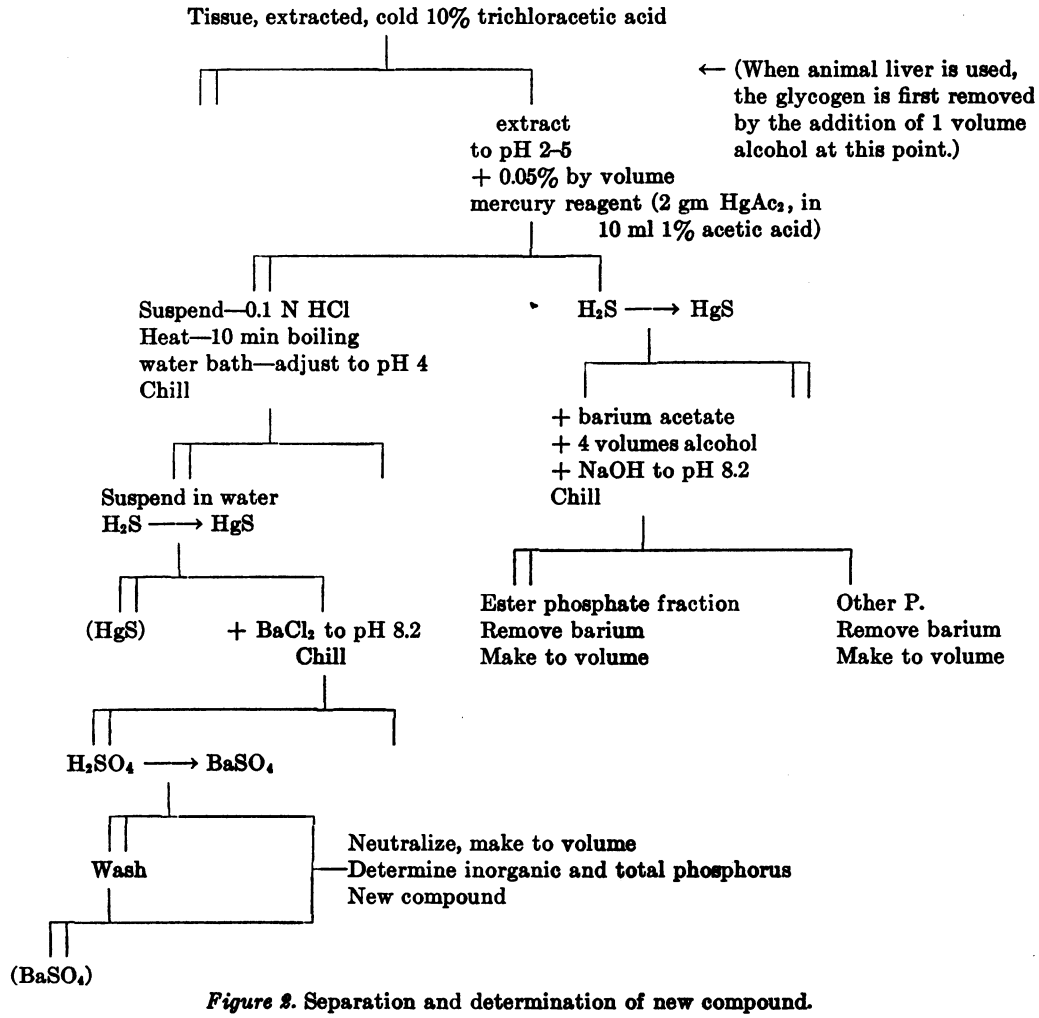

Figure 2. Separation and determination of new compound.

determination. The method, diagrammed in figure 2, is paraphrased as follows:

The new compound forms an insoluble mercury salt and an insoluble barium salt. The trichloracetic acid extract at pH 2 to 4 is treated with mercuric acetate. This precipitates coenzymes, adenosine triphosphate, adenosine diphosphate, adenylic acid, and the new compound. This precipitate is treated with 0.1 N HCl at 100 C for 10 minutes which converts all of the other counts were corrected for the counts due to inorganic phosphate before the organic phosphorus activity was calculated.

While there is no known phosphorylated compound having these properties other than the new compound illustrated in figure 1, additional evidence of the purity and nature of the compound contained in the new compound fraction was desirable. Ascending paper chromatography (in MeOH [80 vol], HCOOH [15 vol], and water

[5 vol]) of the fraction containing the new compound yielded two phosphorus containing spots at R_f 0.24 and R_f 0.42, the last of which was inorganic phosphate. When radioactive samples were chromatographed similarly, the radioactivity was associated with the same two spots. The organic phosphorus in this fraction thus appears to be a single compound. From its method of preparation its mercury and barium salts are insoluble. The phosphorus is bound in an acid resistant form, the hydrolysis curves resembling those of phosphoglyceric acid, which compound is not present in the fractions. The fractions do not give the pentabrom acetone reaction for citrate but give a color with Nessler's solution with a peak absorption in the region of 380 mμ. Inasmuch as all of these are properties associated with the compound isolated by Rapoport and Wagner (1947, 1951), we assume that we are dealing with the same material.

It is obvious that the fraction contains organic phosphorus. It comprises between 0.05 and 0.10 per cent of the organic phosphorus extractable from animal (and bacterial) cells with cold 10 per cent trichloracetic acid and has at least some of the properties ascribed to it by Rapoport and Wagner (1951). Since there is no question but that a compound of this sort exists, the next problem is whether or not it is an important metabolic intermediate.

The activity of the new compound in vivo. Since we have no real knowledge of the immediate precursors from which this compound is derived, the turnover of this compound in rat liver was traced by way of radioactive phosphorus, rather than carbon. The animal provided a useful tool for this study since the compound was known to exist in liver and the streptomycin sensitive reaction has been demonstrated in animal tissue (Umbreit and Tonhazy, 1949). These studies are presented here since they offer information, not yet obtainable on the bacteria, which serves to supplement the bacterial studies reported later. The data apply to the phosphorus turnover only and tell one nothing about the carbon. One may suppose either that the carbon of the compound is turning over actively as well as the phosphorus or that the carbon is inert and the phosphorus is exchanged on it. There is no evidence yet available which will distinguish between these possibilities. The experiments were done by injecting rats with radioactive phosphorus and at some period thereafter killing the animal and grinding the liver in cold 10 per cent trichloracetic acid. (The liver from three rats usually sufficed for one analysis.) The trichloracetic acid extract was fractionated, and specific activity of the fractions compared to that of the total extract (relative specific activity) was taken as evidence of turnover of the phosphorus in the compound.

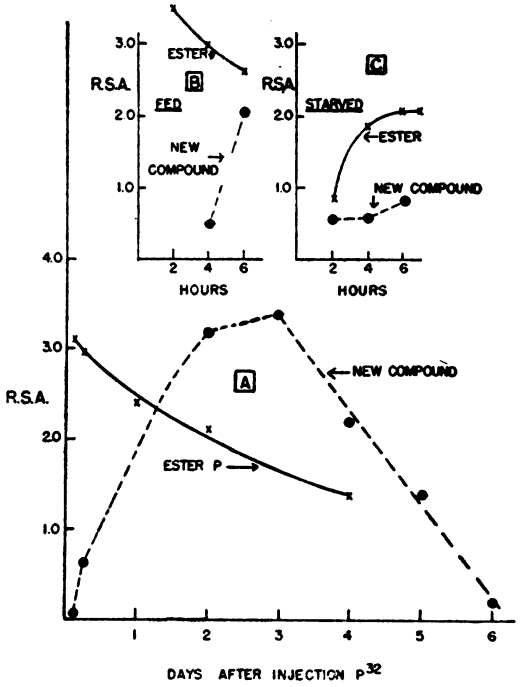

Figure 3. The accumulation of radioactive phosphorus in the phosphorus compounds of rat liver. P^{32} injected at zero time. R.S.A. = relative specific activity = $\frac{\text{specific activity in compounds}}{\text{specific activity of T.C.A. extract}}$. Ester phosphate and new compound as defined in text.

Data from three separate experiments are given in figure 3. Figure 3A shows the development of the relative specific activity in the ester fraction and in the new compound over the course of several days. It is apparent that the phosphorus of the new compound accumulates more slowly than in the ester fraction. Figures 3B and 3C show the results of relatively short term experiments in animals fed *ad lib* and starved (for 24 hours prior to the injection of the radioactive phosphorus and during the 6 hour interval of

the experiment). The livers of the animals in figure 3C were almost devoid of glycogen, and accumulation of radioactivity in the compounds is relatively slower. The data here assembled are sufficient to establish that the new compound is an active metabolic intermediate in the animal, which is the question these experiments were designed to determine.

TABLE 1

Phosphorus distribution and activity in Escherichia coli

Cells of *Escherichia coli*, 120 ml (containing 6.24 g wet weight cells) + 2.5 ml P^{32} (ca 100 μC P^{32}) + 7.5 ml K_2HPO_4 containing 1 mg per ml as carrier. One lot received 12.0 ml water, the other 11.8 ml water + 0.2 ml streptomycin HCl solution containing 100 mg per ml of free base (amount added 20,000 μg = 100 μg per ml final concentration). This was incubated for 30 minutes to allow phosphate and streptomycin to come into equilibrium with cells. At zero time, 41.6 ml 0.05 M pyruvate (pH 7.4) and 16.6 ml 0.5 M fumarate were added to each flask. The contents were shaken in air, 30 C for 3 hours, stopped by the addition of 50 ml of 10 per cent trichloracetic acid and fractionated.

	WITHOUT STREPTOMYCIN		WITH STREPTOMYCIN	
	%		%	
Inorganic P, as % of total	17.3		17.8	
Organic P, as % of total	82.7		82.2	
Fractionation of organic phosphorus—as % total organic				
Found in medium	40.2		40.5	
In trichloracetic acid Extract	12.1		12.8	
New compound	0.089	(1.65)	0.11	(0.24)
Ester	7.0	(2.6)	6.6	(1.4)
Adenylic acid	1.27	(0.25)	0.7	(0.20)
Other	2.6		2.1	
Nucleic acid	16.8	(0.13)	14.3	(0.17)
Cell residue	31.2		32.5	

Figures in parentheses are specific activity of fractions.

While the new compound is involved in the metabolism of the animal, as evidenced by the incorporation of radioactive phosphorus into it, the data presented here indicate that this incorporation is slower than that into the ester fraction, and that, therefore, the material is not as active a part of metabolism as the phosphorylated esters. We have had two experiments of short term duration in which the incorporation of phosphorus into the new compound was faster than incorporation into the ester phosphate. We therefore feel that there are unknown conditions in which the new compound may play a more active part in metabolism, but at present we are not in a position to specify what these conditions may be.

Relation of the new compound to streptomycin inhibition. After some preliminary studies which indicated that the same kind of compound could be obtained from bacterial cells, attempts were made to determine whether this compound was related to streptomycin action. Accordingly, experiments were done with a streptomycin sensitive strain of *E. coli*. Considerable quantities of cells were needed for these experiments which made it difficult to obtain sufficient numbers in the most sensitive state. A compromise was made therefore; the cells were grown on 1 per cent tryptone, 1 per cent yeast extract, 0.5 per cent K_2HPO_4 medium with 1 per cent glucose (rather than 0.1 per cent) and the streptomycin concentration was increased to 20 to 100 μg per ml (the strain is sensitive to 10 μg per ml).

Details of an experiment are given in table 1, from which it is apparent that there is no significant difference in the amount of phosphorus appearing as inorganic or organic forms in the medium, cells, trichloracetic acid extracts, or nucleic acids due to the presence of streptomycin, i.e., the antibiotic is not permitting phosphorus to leak out of the cell or altering in any major way any of the other fractions. The trichloracetic acid extract would contain presumably the metabolically active intermediates. This was separated therefore into fractions containing the new compound, the adenylic acid derived from adenosine triphosphate, the phosphorus associated with the compounds of the Meyerhof-Embden system ("ester phosphorus"), and a fraction containing unknown materials (barium soluble, alcohol soluble). From the amount of phosphorus in each fraction there is again no major change due to the presence of streptomycin. The new compound is present in streptomycin treated cells (in fact it seems to be somewhat higher than in untreated cells). However, presence is not evidence that it has been formed during the incubation period.

For this kind of evidence one must examine the radioactivity of these fractions, data on which are given in parentheses. It will be noted that the specific activity of the new compound fraction is much lower in the presence of streptomycin than that found in the untreated sample, to such an extent, indeed, that there has been an 86 per cent inhibition of the incorporation of radioactive phosphorus into it.

There is also a lowered specific activity in the ester phosphorus fraction (amounting to 40 per cent inhibition of phosphorus incorporation).

TABLE 2

Formation of the new compound by Escherichia coli

Nineteen ml of a suspension of *Escherichia coli* containing 13.4 mg dry weight of cells per ml (2.55 g dry weight of cells) at pH 7, supplied to each flask below:

	1	2	3	4	5	6
Streptomycin, 10 mg/ml (final concentration 20 μg/ml)	—	0.1	—	0.1	—	0.1
After 30 minutes, add P^{32} (in ml)	10	10	10	10	10	10
After 10 minutes, add H_2O (in ml)	40	40	20	20	3	3
0.2 M pyruvate—pH 7 (in ml)	—	—	20	20	20	20
0.5 M fumarate—pH 7 (in ml)	—	—	—	—	17	17
Shaken in air 30 minutes. Reaction stopped, trichloracetic acid, new compound fraction isolated.						
New compound—organic P found, μg	112	61	17.8	1.7	9.9	14.0
Specific activity	0	0	0	0	3.5	0

There is evident no inhibition of the incorporation of phosphorus into the adenylic acid (derived from adenosine triphosphate) or into the nucleic acid.

A further experiment estimated the incorporation of phosphorus into the new compound in the short interval of 30 minutes. The details of the experiment and the results are given in table 2. It is first noted that there is much more of the new compound in cells without substrate than in those in the presence of substrate. This is not newly formed compound since it contains no radioactivity. In the presence of pyruvate (Smith *et al.*, 1949; Umbreit and Tonhazy, 1949) this material apparently decreases in amount, but no new compound is synthesized since no radioactive phosphorus appears in it. In the presence of pyruvate plus a dicarboxy acid (fumarate) there is a similar decrease in the amount of the compound as is seen with pyruvate alone, but the presence of the fumarate now has permitted the incorporation of radioactive phosphorus into the compound. Streptomycin has inhibited this incorporation.

In order to obtain the quantities of cells required for the analytical experiments, air grown *E. coli* cells were employed. These cells possess a system capable of oxidizing acetate which is not sensitive to streptomycin, and the effect of streptomycin on such cells is not demonstrable readily in manometric experiments (*cf* Umbreit *et al.*, 1951). Aliquots of such cells containing 5.25 dry weight of cells were incubated for 30 minutes with 50 μC P^{32} contained in 0.01 M phosphate in order to promote equilibrium between external and internal phosphate. Streptomycin was supplied at several concentrations as indicated (table 3), and the systems were incubated for another 30 minutes to allow the streptomycin to penetrate. At this point pyruvate or pyruvate + oxalacetate was added, and the suspension was incubated in air with shaking for 60 minutes when the systems were stopped by the addition of sufficient trichloracetic acid to render the final concentration

TABLE 3

Formation of the new compound by Escherichia coli, air grown cells

SUBSTRATE ADDED	STREPTOMYCIN	SPECIFIC ACTIVITY OF NEW COMPOUND
None	0	3.9
Pyruvate (1 mM)	0	3.8
Pyruvate (1 mM)	50 μg/ml	3.3
Pyruvate (1 mM) + Oxalacetate (2 mM)	0	17.3
Pyruvate (1 mM) + Oxalacetate (2 mM)	50 μg/ml	5.0
Pyruvate (1 mM) + Oxalacetate (2 mM)	25 μg/ml	1.7
Pyruvate (1 mM) + Oxalacetate (2 mM)	50 μg/ml (2 hours)	3.0

10 per cent. The extraction was carried out at room temperature for 3 days, the fraction containing the new compound isolated from the trichloracetic acid extract, and the specific activity of the organic phosphorus determined. The data obtained are given in table 3. Apparently during the hour of incubation before the substrates were added, some radioactive phosphorus was incorporated into the new compound resulting in a specific activity of 3 to 4. Subsequent incubation with pyruvate (with or without streptomycin) did not alter this activity significantly. Incubation with oxalacetate and pyruvate, however, increased the specific activity about fourfold. In the presence of streptomycin these substrates did not permit the increased radioactivity. The streptomycin was not merely slowing the rate at which radioactivity was incorporated into this fraction since incubation for an additional hour (see table 3) with streptomycin resulted in no increase in activity. The specific activities of the streptomycin containing samples (varying from 1.7 to 5.0) are not significantly different, largely due to the relatively small amount of phosphorus found with these samples (of the order of 25 μg) which make precise estimation of the specific activity difficult.

Using the same cellular suspension, but in one-third the amount, a series of isolations was made after varied incubation periods with oxalacetate and pyruvate. The precision of measurement was somewhat less in the data of table 3, but the activity of the fraction containing the new compound rose steadily reaching its peak at 90 minutes, and thereafter losing activity so that by 5 hours it was virtually the same as the endogenous. The amount of the compound per cell had a tendency to drop during the rapid oxidation period and to return to the endogenous values (or above) when oxidation slowed.

DISCUSSION

It seems apparent from the data of these experiments that the compound is formed when pyruvate and fumarate or oxalacetate are present and is not formed when only pyruvate is supplied. However, it is also apparent that the retention or breakdown of the compound within the cell is influenced by the presence of both external substrate and streptomycin so that the situation is far from simple. We therefore feel that it is safe only to conclude that streptomycin inhibits the formation of the new compound, but that it is not apparent what role the new compound plays in bacterial or animal metabolism. At least two possibilities are available from present data. First, the compound might be the actual intermediate entering the terminal respiration system, comparable to citrate in the Krebs cycle. Second, the compound might be a "coenzyme" whose presence is necessary for other reactions to proceed. A great deal of study will be required before the role of this compound in metabolism can be evaluated. At present it is of interest that such an intermediate exists and that its formation is inhibited by streptomycin.

Some attempts have been made to isolate the compound in sufficient quantity to perform metabolic experiments with it. It can be isolated readily from rat liver, but the small quantity present (approximately 0.3 μg P per g wet weight) precludes this source as a starting material. Dried horse liver and fresh beef liver also contain the compound, but fractions containing it are contaminated by a brown waxy material which interferes with metabolism and which we do not know yet how to remove.

SUMMARY

A seven-carbon phosphorylated compound, 2-phospho-4-hydroxy-4-carboxy-adipic acid, is shown to be a metabolic intermediate as determined by the incorporation of radioactive phosphorus. In *Escherichia coli* it is formed on the addition of pyruvate and oxalacetate or fumarate, and its formation is inhibited markedly by streptomycin.

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