THE FORMATION OF d-GLUCONIC ACID BY BACTERIUM SAVASTANAI SMITH.

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Dr. Erwin F. Smith found that the olive tubercle organism, Bacterium Savastanoi, studied and named by him, forms an acid when grown aerobically in the presence of certain sugars. Some time ago Dr. Smith requested me to attempt the identification of the acid formed in the presence of glucose.¹ To that end he supplied me with four flasks of these organisms grown during five months at room temperature upon a medium consisting of 14 grams of Witte peptone, 28 grams of calcium carbonate, 20 grams of Merck's dextrose and 1000 cc. of filtered tap water. It is therefore to Dr. Smith's courtesy that I am indebted for the opportunity to make the study herein recorded.²

The calcium carbonate, found to be free from insoluble organic calcium salt, was removed from the culture by filtration. The clear filtrate was concentrated to a thin syrup. It was set aside and the walls of the vessel occasionally rubbed with a glass rod. In this way an abundant, white, cauliflower-like mass developed, that under a hand lens could be seen to consist of aggregates of fine needles. The crystals were drained under pressure from the mother liquor before recrystallizing from hot water. The mother liquor from the flasks first used reduced Fehling's solution powerfully, the crystals did not. The flask used last, which held a

Formation of d-Gluconic Acid

culture two months older, contained no reducing material. By repeated recrystallizations white crystals were easily obtained without the use of bone-black. They showed great tendency to form supersaturated solutions, so that it was often necessary to inoculate with a few crystals to obtain a satisfactory crystallization. The purified preparation was then dried under diminished pressure over sulphuric acid at room temperature. When at constant weight it was further dried in an air bath at a temperature rising gradually to 115° C., when it lost nothing more, so that if water of crystallization were present, as claimed by some, it must have been removed at room temperature under diminished pressure over sulphuric acid. The calcium content was then determined by incinerating and heating to constant weight. It proved to be 9.36 per cent. This corresponds to anhydrous calcium d-gluconate which contains theoretically 9.35 per cent calcium. Thereupon the acid was set free by precipitating the calcium with oxalic acid, and removing the calcium oxalate. The solution was extracted with ether to remove any slight excess of oxalic acid, and evaporated to a syrup. There was some indication of crystallization in the syrup, although crystals were not abundant. d-Gluconic acid does not crystallize, but the crystallizing lactone forms easily. From the free acid the cinchonine salt was prepared. This had the characteristic appearance and solubilities of cinchonine d-gluconate. When the acid was treated with a faintly acid solution of ferric chloride a fine yellow color was obtained, a reaction which d-gluconic acid shares with a number of other oxy-acids. From the calcium salt the hydrazide was prepared as recommended by Fischer. Here, too, the tendency to form supersaturated solutions delays crystallization. After three recrystallizations from hot water the hydrazide showed the characteristic behavior and melting point of d-gluconic acid hydrazide. The hydrazide dissolved in strong sulphuric acid gave a red coloration with ferric chloride, as shown by Bülow’s Reaction.
The optical activity was determined as recommended by Fischer.² Twenty-five centigrams of the calcium salt gave a rotation of +1.50° in a 100 mm. tube. Fischer obtained +1.55° under these conditions.

There can therefore be no doubt that the acid obtained from the cultures was d-gluconic acid. This acid has been obtained before as the result of the action of other organisms upon glucose;² but this, so far as I am aware, is the first record of its production by a pathogenic organism.

At the beginning of this investigation it was assumed that the reducing substance remaining in the mother liquors after the crystallization of the calcium d-gluconate was glucose. Since Boutroux,⁸ however, has described an organism which is capable of making an oxy-gluconic acid with reducing power from either glucose or d-gluconic acid, it is possible that the reducing material in the cultures of Bacterium Savastanoi Smith is a further oxidation product of d-gluconic acid. Some of the filtered culture fluid, consequently, was treated with 95 per cent alcohol until further addition of alcohol caused no more precipitation. The crumby white precipitate formed was filtered off, redissolved in a small volume of water, and reprecipitated with alcohol. The precipitate then no longer reduced Fehling's solution. All the reducing material had passed into the alcoholic filtrates. The alcohol was removed under diminished pressure at a temperature below 50°. A sample of the solution after having been freed from alcohol in this way was readily fermented by yeast so that its reducing power quite disappeared. It still contained a little calcium d-gluconate. From the unfermented solution the osazone was obtained in the usual way. The osazone was separated from the hydrazone of d-gluconic acid by means of its greater insolubility in hot water. It was re-crystallized until its melting point remained constant at 204°. There can therefore be no doubt that the reducing substance was unfermented glucose. This was subsequently confirmed by the fact, already mentioned, that older cultures lost their reducing power.

¹ Ber. d. deutsch. chem. Gesellsch., xxiii, p. 2611.
² The literature has been collected by Lippmann: Chemie der Zuckerarten, pp. 323 and 431.
⁸ Boutroux: Sur une fermentation acide du glucose, Compt. rend. de l'Acad. des sci., cii, p. 924; cxi, p. 185.
The loss of reducing power did not seem to be accompanied by any considerable loss of d-gluconic acid as far as can be judged from the large yields of calcium d-gluconate obtained from these old cultures. From a seven-months-old culture which no longer contained any of the 20 grams of glucose originally put into it, 15.93 grams of calcium d-gluconate were obtained. As this salt could not be separated quantitatively from the mother liquor, the amount remaining in solution was estimated by determining the calcium in the mother liquor. Before incineration the solution was boiled and filtered to remove any calcium bicarbonate that may have been in solution. The calcium in solution corresponded to 7.33 grams of calcium gluconate, so that 21.32 grams of d-gluconic acid had been formed corresponding to 17.02 grams of glucose. The deficit was less than 3 grams of glucose. The glucose used was Merck’s anhydrous glucose. In preparing the culture medium it was taken directly from the bottle without further drying. It may not have been quite anhydrous and, if so, the amount of glucose unaccounted for may be even less. At any rate it is justifiable to conclude that only a little glucose was converted into anything other than d-gluconic acid. There may be a small error in this calculation due to the fact that the assumption is made that the calcium remaining in the mother liquor is combined with d-gluconic acid and that other soluble calcium salts are not present. Certainly no other acid was found.

The amount of energy liberated by the oxidation of glucose to d-gluconic acid is very considerable. The heat of combustion of glucose is 673.7 Cal. I have not been able to find data on the heat of combustion of d-gluconic acid; but that of the lactone of l-gluconic acid is 615.3 Cal. The heat of combustion of d-gluconic acid will be different, though probably not very much. For want of better data, I have assumed that it is the same. Then the heat of reaction in the conversion of glucose into d-gluconic acid is probably about 58 Cal. or 8.6 per cent of the total energy obtainable by the complete combustion of glucose. This is of the same order of magnitude as some other fermentations such as the formation of alcohol.\(^2\) Inasmuch as about 17.02 grams of glucose have

\(^1\) Lippmann: \textit{op. cit.}, p. 1742.

been oxidized, it follows that about 5.48 Cal. have been liberated by the organisms in a single flask in the course of seven months or an average of .026 Cal. per day, provided we assume that it is permissible to consider only the simplest fermentation equation. That such calculations are very inaccurate either because they disregard the heats of solution or because the fermentation equations are incomplete as well as for a variety of other reasons has been shown by Rubner.¹ Still for the determination merely of the order of magnitude of the energy converted in the formation of d-gluconic acid it is accurate enough, particularly as it represents minimum not maximum values. It is possible that there is still another source of error in this calculation due to the fact that all the glucose may have been converted some days or even weeks before these determinations were made. However, as this error if present would make the per diem values smaller, not larger, it can not impair the argument. Moreover, this calculation does not take into consideration the 3 grams of glucose unaccounted for, which were probably also oxidized to furnish heat as they could not be stored as reserve carbohydrate in the small amount of micro-organisms present. Even an accurate knowledge of the energy converted would be of very little value without a knowledge of the mass of living matter concerned in the process. To weigh the micro-organisms in liquid culture media accurately is as yet impossible.² Results, sufficiently accurate for the approximations necessary in this discussion, were obtained thus:—The organisms were decanted from the lime salts present in the medium. The remaining lime salts were suspended in much water and dissolved slowly with acid, without using an excess. The solutions were then filtered through asbestos in perforated crucibles. The residues were washed, dried, and weighed. The filtrates were centrifugated at high speed and the weight of the washed, dried sediment added to that of the Gooch crucibles. The dry weight of the micro-organisms was thus found to be 0.0087 gram, which can not be regarded as more than a very rough approximation. If we assume the organism to contain not over 85 per cent

Formation of d-Gluconic Acid

water\(^1\) the live weight would be .058 gram. We must, however, further take into consideration that probably all organisms were not active at any one time. The organism is aerobic and perhaps only the surface pellicle was actively oxidizing. Those organisms which had dropped beneath the surface were probably not active, though they may have been alive. If such organisms were dead they might have been autolyzed setting enzymes free in the medium to continue oxidation outside the cells. This possibility deserves consideration in view of recent studies on bacterial oxidizing enzymes surviving the bacteria themselves.\(^2\) No oxidation was found in a preliminary experiment with filtered culture containing an antiseptic, toluol. Possibly experiments of longer duration might reveal it. Moreover, in view of the great difficulty with which such intracellular enzymes are separated from the cells, and further, in view of their great instability when separated, any considerable oxidation by this means seems improbable. At any rate that it may occur remains to be proved. For non-oxidizing enzymes, a post-mortem action on the medium has been demonstrated in the ripening of cheese.\(^3\)

The amount of material metabolized and the amount of chemical energy converted is therefore very large when compared with the corresponding processes of higher forms such as germinating seeds or man. The Bacterium Savastanoi Smith under the conditions of these experiments according to this approximate calculation converts daily an amount of energy equivalent to at least 448.4 Cal. per kilo of organisms. Men require about 35 Cal. per kilo per day. The same fact has been brought out by Stoklasa. He found that 100 grams of dry Clostridium butyricum yielded 2.13 grams of carbon dioxide per hour; Bacterium Hartlebii 2.89 grams, sugar beet root 0.006 grams.\(^4\) Rubner has also repeatedly drawn attention to this phenomenon and published fundamental investiga-

\(^1\) M. Rubner: Das städtische Siedlswagen und seine Beziehung zur Flussverunreinigung, Arch. f. Hygiene, xlvi, p. 41.
\(^3\) O. Jensen: Landwirtschaftliches Jahrbuch der Schweiz, 1906, p. 303.
The significance of these great quantitative differences between the metabolism of micro-organisms and that of larger forms of life are not yet quite clear. An attempt to offer an explanation for these differences and to point out their relations to theories of fermentation was made at the time this paper was presented. Its publication is reserved for another communication.

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