ON THE EFFECT OF ARSENATE ON BLOOD GLYCOLYSIS

A Correction

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In their paper on the effect of arsenate on blood glycolysis Morgulis and Pinto (1932) discuss among other data those of Engelhardt and Braunstein (1930) and of Braunstein (1931). To our experimental technique and theoretical arguments some objections are raised by Morgulis and Pinto, indicating that they overlooked several points stated in our work and in consequence have misinterpreted it. Some explanatory remarks appear therefore necessary.

1. Morgulis and Pinto write: "Braunstein using the Fiske-Subbarow method for the phosphate determination depends upon rapid reading of the colors to avoid errors due to the presence of arsenate, the extra color development from arsenate presumably being a slower process." This procedure of quantitative determination is termed "uncertain and indeed . . . very uncritical." These authors fail to notice the fact mentioned in both the articles by Engelhardt and by Braunstein that a modification of the Fiske-Subbarow method was used, as described by Braunstein (1928). Further it is explicitly stated in both of our papers that a correction for arsenate was applied to the phosphate determinations.

The method employed by us for the estimation of inorganic phosphate is essentially an adaptation of Lohmann and Jendrassik's (1926) modification of the Fiske-Subbarow method for use with smaller amounts of material (the final volume of the reaction mixture being 2.5 to 5 cc.); further improvements are introduced concerning the technique of the total and acid-soluble phosphorus determinations. The procedure of Lohmann and Jendrassik involves the use of a molybdate solution of higher acidity than does
the original method of Fiske and Subbarow and the warming of the material on a water bath at 37° for 4 or 5 minutes to complete the color development. Immediately on removal from the water bath and cooling, a solution of arsenate treated according to this technique displays a very slight blue coloration; up to m/600 to m/300 solutions this color is too faint for colorimetry; on standing at room temperature the color gradually darkens, and in the course of several hours grows deeper by far than the color developed by

**TABLE I**

*Colorimetric Determination of Phosphate in Standard Solutions of Potassium Phosphate in Presence of Arsenate*

(Illustrating the correction to be taken into account and the rôle of the time factor.)

Test solutions plus reagents were warmed to 37° for 4 minutes; then cooled under the tap. Room temperature, 14°.

<table>
<thead>
<tr>
<th>Time after removal from water bath</th>
<th>m/600 Na₃H₂AsO₄</th>
<th>m/600 KH₂PO₄</th>
<th>m/600 KH₂PO₄ + m/600 Na₃H₂AsO₄</th>
<th>0.002 M KH₂PO₄</th>
<th>0.002 M KH₂PO₄ + m/600 Na₃H₂AsO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>min.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0.114</td>
<td>0.114</td>
<td>0.141</td>
<td>0.142</td>
</tr>
<tr>
<td>2</td>
<td>0*</td>
<td>0.117</td>
<td>0.118</td>
<td>0.145</td>
<td>0.144</td>
</tr>
<tr>
<td>4</td>
<td>0.004</td>
<td>0.120</td>
<td>0.120</td>
<td>0.146</td>
<td>0.146</td>
</tr>
<tr>
<td>6</td>
<td>0.004*</td>
<td>0.122</td>
<td>0.122</td>
<td>0.148</td>
<td>0.148</td>
</tr>
<tr>
<td>8</td>
<td>0.008*</td>
<td>0.126</td>
<td>0.126</td>
<td>0.150</td>
<td>0.150</td>
</tr>
<tr>
<td>10</td>
<td>0.012*</td>
<td>0.130</td>
<td>0.142</td>
<td>0.152</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
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<td></td>
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<td>15</td>
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<td>20</td>
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</table>

* Color too pale for exact colorimetric estimation.

an equivalent amount of phosphate. The color developed in mixtures of phosphate and arsenate is additive for any given moment of time. The rate of color development in m/600 arsenate as well as in mixtures with phosphate is shown by the results of an experiment performed in order to work out a correction graph for m/600 arsenate in phosphate determinations (Table I).

It may be seen that the correction is less than 0.005 mg. per cc.
of $P_2O_5$, provided the colorimetric reading be taken within 5 minutes after removal from the water bath, the moment of the reading, of course, being exactly noted. As the concentration of inorganic phosphate in our experiments usually has the order of magnitude of 0.060 to 0.200 mg. per cc., or 0.001 M to $M/300$, the correction reaches 10 per cent at most.

As opposed to this, Morgulis and Pinto use the Kuttner and Cohen (1927) method of phosphate determination, likewise deducting a correction for arsenate. They state that "the color produced by As is more than 3 times as intense as that produced by an equivalent amount of P." This would mean that the colorimeter reading obtained with a $M/600$ solution of arsenate corresponds to about 0.500 mg. per cc. of $P_2O_5$, with 0.002 M arsenate (a concentration used in some experiments by Morgulis and Pinto) to more than 1.500 mg. per cc. of $P_2O_5$. The correction, therefore, would be 100 to 300 times as large as in our method; in fact, it would amount to three- to tenfold the actual content of inorganic phosphate to be expected in blood samples.

2. Our hypothesis as to the possibility of hexosearsenate formation is discarded by Morgulis and Pinto on account of their failure to observe by means of their colorimetric method a diminution in the total color evolution that would correspond to an esterification of arsenate equivalent in amount to the phosphate liberated.

We, of course, never held the opinion and never asserted that stable hexosearsenates would be synthesized and accumulated by the glycolyzing blood cells. Only an intermediate formation of a highly unstable compound followed under conditions approximating the physiological state by immediate breakdown to inorganic arsenate and products of hexose disintegration is to be expected.¹ Neither are the phosphorus compounds that are disintegrated in non-glycolyzing blood and synthesized during glycolysis hexosephosphoric esters. It was shown by our experiments (Braunstein, 1931) that they belong partly to the pyrophosphate, partly to the diphosphoglycerate fraction. Their

¹ This assumption is corroborated by experiments of Braunstein and Levitov (Braunstein, A., and Levitov, M., Naturwissenschaften, June (1932); Biochem. Z., in press) on the esterification of inorganic arsenate by fermenting yeast preparations.
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formation apparently is connected or coupled in some way, or other with the intermediate phosphorylation of the blood sugar during glycolysis. According to our view, the action of arsenate depends on its impeding the esterification of the hexose with phosphate by "competitive inhibition" and thus preventing the resynthesis of the organic phosphorus compounds mentioned above, whereas their disintegration by the phosphatases of the blood goes on unimpaired.

3. Our statement that arsenate does not produce any acceleration of the breakdown of phosphorus compounds in non-glycolyzing blood, where there is no overlapping of the hydrolytic process by resynthesis, is misunderstood by Morgulis and Pinto as indicating that we negate the possibility of the phosphatase and the glycolysis enzyme systems being affected independently. It is evident from our publications that we always maintained the opposite view. The fact observed by Morgulis and Pinto that arsenate always promotes the liberation of inorganic phosphate regardless of whether this is accompanied by an acceleration of glycolysis or a partial inhibition (as in dog blood), is due to the precluding of resynthesis and corroborates (as does the similar effect of CaCl₂ in the experiments of Roche and Roche (1929)) the view of Engelhardt and Braunstein that the reesterification of phosphate is a phenomenon connected with glycolysis, but not a necessary condition of the latter.

4. The explosive increase of the inorganic phosphate during hemolysis alluded to by Morgulis and Pinto as "the maximum liberation of phosphate," is due to the breakdown of pyrophosphate, first investigated by Engelhardt (1930). It is followed by a comparatively slow disintegration of other phosphorus compounds (chiefly diphosphoglycerate). To this latter process our statement relates that arsenate does not affect the hydrolysis of phosphate esters in hemolyzed blood.

5. Morgulis and Pinto assign to Macfarlane the assertion that arsenate stimulates phosphatase activity. In fact Macfarlane (1930) observed that arsenate does not influence the liberation of inorganic phosphate from hexosediphosphate by cozymase-free yeast preparations. Only when a simultaneous fermentation of the hexosephosphate is taking place, there is an increase of the rate of dephosphorylation by arsenate. Macfarlane conclude
that the action of arsenate is not due to a direct effect upon the hydrolysis of hexosediphosphate, but is a phenomenon of complex character, being connected somehow with the action of cozymase (and possibly with some further factor contained in the coenzyme fraction). In a recent survey of the biochemistry of alcoholic fermentation, Harden cited in Nord-Weidenhagen (1932) sustains the view, based mainly on the data of Macfarlane, that the "arsenate effect" depends on the activation of a special enzymic mechanism performing the direct fermentation of hexosediphosphate in the presence of cozymase, rather than on the stimulation of the hydrolytic enzyme phosphatase.

Since hemolyzed blood contains cozymase and is capable of glycolyzing added hexosediphosphate to an appreciable extent, the stimulation by arsenate of phosphate liberation from hexosediphosphate in hemolyzed blood, observed by Morgulis and Pinto, is by no means a decisive proof that arsenate acts through direct stimulation of hexosephosphatase. The objections put forward by Macfarlane and by us against such an interpretation of the acceleration of hexosephosphate breakdown by arsenate apply to these experiments as well. With the glycolytic enzyme system of erythrocytes totally hemolyzed at low temperature, Meyerhof (1932) observed no stimulating effect of arsenate on the breakdown of hexosediphosphate.

To conclude with, it must be stated that the greater increase in inorganic phosphate and the slower rate of glycolysis observed by the authors in physiological NaCl solution than in Locke's solution is readily explained by the damaging action of dilution with normal saline on the glycolytic system of blood cells, described by Engelhardt and Braunstein (1930). Figs. 4 and 5 of the paper of Morgulis and Pinto (1932) show the additive results produced by the specific action of arsenate and by the damaging of the blood cells with normal saline.

The observation of Morgulis and Pinto, in itself interesting, that dog blood responds to arsenate by a diminished glycolysis, is

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1 The method of hemolysis used by these authors is not altogether satisfactory; the addition of an equal volume of distilled water to blood cells produces very incomplete cytolysis. Either considerably larger amounts of distilled water or ether-saturated water should be employed in order to insure total laking of the blood.
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perhaps connected with the well known fact of its relatively weak glycolytic activity (Engelhardt and Ljubimowa, 1930) and with the rapid decline of the rate of the glycolytic process, illustrated by Fig. 3 in the paper of Morgulis and Pinto. This seems to indicate that the glycolytic system of the dog erythrocytes is rather unstable. It might be that it is more susceptible to the toxic influence of arsenate than that of rabbit erythrocytes which also are damaged by higher concentration of arsenate (Braunstein, 1931). The observations of Meyerhof (1921, 1927) on the arsenate effect in muscle and muscle preparations offer another instance that the action of arsenate, especially with regard to the concentrations producing stimulation, depends to a considerable extent upon the nature and condition of the individual glycolytic system. The toxic effect of course must have been especially marked in those experiments of Morgulis and Pinto where the glycolytic system had been damaged by normal saline or by prolonged incubation (4 hours). In fact there is no inhibition of glycolysis by arsenate apparent in dog blood cells incubated for 2 hours, to judge from Fig. 2 of Morgulis and Pinto; there seems rather to be a slight stimulation.

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