The measurement of the activity of an enzyme in tissue extracts, in preparations of differing purity, or in biological fluids may properly be made by determining the amount of substrate changed in a stated time, provided that the change is within the initial zero order portion of the reaction (1-3). Under this provision and in the presence of suitable activators the amount of substrate changed in the stated time is directly proportional to enzyme concentration (1). However, the use of this measure of reaction velocity requires constant attention to experimental conditions so that the amounts of the substrate changed do indeed fall within the initial portion of the reaction. In addition, since most enzyme reactions are of zero order only during the first 5 or 10 per cent change of the substrate, the use of this measure involves a considerable wastage of substrate, sometimes of expensive or scarce material.

The present paper shows how the amount of substrate changed at any stage of the reaction may be properly employed for the measurement and comparison of enzyme activities. The relationship underlying this mode of measurement is developed mathematically and is then illustrated and confirmed by data on the activity of serum phosphohexose isomerase.

EXPERIMENTAL

Human sera were used as the source of phosphohexose isomerase activity. The method of determining phosphohexose isomerase activity by following the formation of fructose-6-phosphate from glucose-6-phosphate has been previously described (4). In the present study, the activities of phosphohexose isomerase were determined at the optimal pH range 7 to 8, 37°C, and at a substrate concentration of 0.002 M glucose-6-phosphate. This concentration is considerably below the maximal range (4).

Results

Theoretical—The principle of measurement to be described here involves the following operations. The amount of substrate which is changed in a
stated time, $T$ minutes, is determined at various concentrations of an arbitrarily defined reference preparation, $E$, of the enzyme, and a reference curve is constructed on the basis of these data. The change in the substrate may range up to 100 per cent of its total initial concentration.

The activity of any unknown preparation, $E'$, of this enzyme is then ascertained (a) by arbitrarily choosing a convenient change, $S_1$, in the substrate produced in $T$ minutes by the concentration, $E_a$, of the reference enzyme preparation; (b) by determining the amount of substrate, $S_2$, changed in $T$ minutes by a concentration, $E_b'$, of the unknown enzyme preparation; (c) by noting from the reference curve that concentration, $E_b$, of the reference enzyme preparation which would produce the observed change of $S_2$ in the stated time, $T$. The activity of the unknown enzyme preparation is then obtained from Equation 1

$$E_a' = \frac{E_b'}{E_b} \times E_a$$

where $E_a'$ is defined as the concentration of the unknown preparation that would produce the standard change, $S_1$, in the stated time, $T$.

Equation 1 is derived as follows: For those enzyme preparations which contain suitable activators and do not contain inhibitors, the time-change curve has the same mathematical form at different concentrations of enzyme (1), and the following relationship, first observed by Arrhenius (5) and by Osterhout (6), holds

$$K = \frac{1}{t_x E}$$

where $t_x$ is the time necessary to produce a change, $S_2$, in the substrate at the concentration, $E$, of a reference enzyme preparation. For a stated change, $S_2 = S_1$, the following expression would hold

$$K_1 = \frac{1}{E \times t_1}$$

where $t_1$ is the time necessary for the change, $S_1$, to occur at the enzyme concentration, $E$. The concentration, $E_a$, of the reference enzyme preparation needed to effect the stated change, $S_1$, in an arbitrarily chosen specific time, $T$, would be expressed by Equation 4.

$$K_1 = \frac{1}{E_a \times T}$$

Combination of Equations 3 and 4 results in the following expression

$$E_a = \frac{E \times t_1}{T}$$
Let us now consider a non-reference, unknown preparation, \( E' \), of the enzyme which, as has already been assumed, has the same mathematical form for the time-change curve. Since the time, \( n t_1 \), will be required to effect the stated change, \( S_1 \), in the substrate, then, as in Equation 3,

\[
K_1' = \frac{1}{n t_1 \times E}
\] (6)

where \( K_1' \) is a constant representing the proportionality of reaction velocity and enzyme concentration in the second, non-reference preparation. The concentration of enzyme, \( E_a' \), of this preparation necessary to effect the stated change, \( S_1 \), in the substrate in the stated time, \( T \), will therefore be, as in Equation 5,

\[
E_a' = \frac{E \times n t_1}{T}
\] (7)

Combination of Equations 5 and 7 yields

\[
\frac{E_a'}{E_a} = n
\] (8)

In other words, the ratio of the concentration of the non-reference to that of the reference preparation necessary to effect the stated change in a stated time is a constant and is equal to the ratio of the reaction velocity of the reference to that of the non-reference enzyme.

It may also be shown in a similar manner that

\[
\frac{E_a'}{E_b} = n
\] (9)

where \( E_b \) and \( E_b' \) are, respectively, the concentrations of enzyme in the reference and in the non-reference unknown preparation, expressed as cc. per cc. of reaction mixture, needed to effect an observed change of \( S_2 \) in the chosen stated time, \( T \). Hence

\[
\frac{E_a'}{E_b} = \frac{E_b'}{E_a}
\] (10)

By transposition, this becomes

\[
E_a' = \frac{E_b'}{E_b} \times E_a
\] (11)

where, as previously noted, \( E_a \) and \( E_a' \) are the concentrations, respectively, of the reference and unknown preparations that produce the stated change, \( S_1 \), in the stated time, \( T \). The activity of the unknown preparation may be more directly described as the reciprocal of \( E_a' \).

\[
\text{Activity of unknown preparation} = \frac{E_b}{E_b'} \times \frac{1}{E_a}
\] (11)
This expression of the enzyme activity is thus independent of the extent of change in the substrate.

Identity of Form of Time-Change Curve for Phosphohexose Isomerase Activity—The development of Equations 1 and 11 involves the assumption that the form of the time-change curve is the same for different preparations of a particular enzyme or for different dilutions of the same preparation. Assurance that the form of the function does not change with variation in concentration of the enzyme is given by the finding that the ratios of the reciprocals of the time necessary to produce a stated change in the substrate are the same at different points in the course of the time-change curve (1). It was previously found that this relationship held for different dilutions of serum phosphohexose isomerase (4). Table I presents several typical experiments which demonstrate that the form of the time-change function is the same for different sera.

Reference Curve for Serum Phosphohexose Isomerase Activity—The amounts of fructose formed as fructose-6-phosphate per cc. of reaction mixture in 30 minutes from 0.002 M glucose-6-phosphate at optimal pH (7 to 8) and 37° were determined at various concentrations of an arbitrarily chosen serum (Serum 1). The activity of this serum was such that 20 γ of fructose were formed at a serum concentration of 0.003 cc. per cc. of reaction mixture, 30 γ at a concentration of 0.0045 cc., 40 γ at a concentration of 0.006 cc., and 50 γ at a concentration of 0.008 cc. It was possible to obtain additional and confirmatory points on this curve with any other serum. For example, with a second serum the comparable amounts of fructose were formed, respectively, at 0.0018, 0.0027, 0.0037, and 0.0047 cc. per cc. of reaction mixture. The average value for the ratios of these concentrations to those of the first serum was 0.60. The concentrations of the second serum necessary to form 60, 80, and so on up to 130 γ of fructose as fructose-6-phosphate in 30 minutes were then divided by 0.60 to give additional points on the first curve.

Fig. 1 represents the reference curve obtained on the basis of determinations with four sera. The phosphohexose isomerase activity of any serum could then be expressed in terms of the reference serum in the following manner. The standard change, \( S_1 \), was arbitrarily designated as 25 γ. \( E_a \), the concentration of the reference enzyme, necessary to produce this change in 30 minutes, was 0.0037 cc. of serum per cc. of reaction mixture. In accordance with Equation 11,

\[
\text{Activity of unknown preparation} = E_b \times \frac{1}{E_a \times E_b'}
\]

The activity of various sera could then be obtained by multiplying \( E_b \), the concentration of the reference serum that would be necessary to produce
the observed change, by 6700 when the concentration of serum was 0.04 cc. per cc. of reaction mixture and by 26,800 when the concentration was

### TABLE I

**Identity of Form of Time-Change Curve for Phosphohexose Isomerase Activity of Different Sera**

Concentration of glucose-6-phosphate, 0.002 M; of serum, 0.04 cc. per cc. of reaction mixture; of Na diethyl barbiturate buffer, 0.012 M, or of Michaelis acetate-diethyl barbiturate buffer, 0.057 M; temperature, 37\(^\circ\); pH, 7.4 to 7.5.

<table>
<thead>
<tr>
<th>Fructose formed as F-6-P per cc. reaction mixture</th>
<th>Serum Au (Reciprocal of time)</th>
<th>Serum pool (Reciprocal of time)</th>
<th>Serum Br (Reciprocal of time)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount</td>
<td>Fraction of maximum*</td>
<td>As ratio to that of Serum Au</td>
<td>As ratio to that of Serum Au</td>
</tr>
<tr>
<td>20</td>
<td>14</td>
<td>0.100</td>
<td>0.167</td>
</tr>
<tr>
<td>40</td>
<td>28</td>
<td>0.0454</td>
<td>0.0769</td>
</tr>
<tr>
<td>60</td>
<td>42</td>
<td>0.0265</td>
<td>0.0465</td>
</tr>
<tr>
<td>80</td>
<td>56</td>
<td>0.0174</td>
<td>0.0286</td>
</tr>
<tr>
<td>100</td>
<td>70</td>
<td>0.0119</td>
<td>0.0188</td>
</tr>
<tr>
<td>120</td>
<td>84</td>
<td>0.0078</td>
<td>0.0098</td>
</tr>
</tbody>
</table>

* This is based on the maximal amount of fructose-6-phosphate, namely 144 \(\gamma\) per cc. of the reaction mixture, that can be formed at equilibrium when the mixture is 60 per cent glucose-6-phosphate-40 per cent fructose-6-phosphate.

0.01 cc. The activity is expressed as units with the dimensions of cc\(^{-1}\).

For example, the activity of Serum M. F. averaged 318 units and was 4.3 times the activity, 74 units, of Serum B. M. (Table II).
It should also be possible to establish similar reference curves for other serum enzymes or for enzyme preparations made from cells or tissues. In the latter case, $E_r$, the concentration of the particular reference enzyme necessary to produce a standard change in a stated time, would be expressed as the weight of protein per cc. of reaction mixture, and the activity of any preparation of this enzyme would be expressed in arbitrary units with the dimensions of $\gamma^{-1}$ or mg.$^{-1}$. Translation of arbitrary units into more absolute terms could be made when a highly purified or crystalline preparation of an enzyme became available, since the abscissas of the reference curve could then be expressed as weights of enzyme protein.

### Table II
Comparison of Enzyme Activities at Stages beyond Zero Order Portion of Reaction

<table>
<thead>
<tr>
<th>Serum</th>
<th>Observed change, $S_r$, in substrate at 0.01 cc. per cc. reaction mixture</th>
<th>Ratio of change at 0.04 cc. to 4 times that at 0.01 cc.</th>
<th>Activity according to Equation 11 at 0.01 cc. per cc. reaction mixture</th>
<th>Activity according to Equation 11 at 0.04 cc. per cc. reaction mixture</th>
<th>Ratio of activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. M.</td>
<td>19</td>
<td>65</td>
<td>0.86</td>
<td>74</td>
<td>74</td>
</tr>
<tr>
<td>G. B.</td>
<td>22</td>
<td>73</td>
<td>0.83</td>
<td>85</td>
<td>85</td>
</tr>
<tr>
<td>N. T.</td>
<td>27</td>
<td>87</td>
<td>0.81</td>
<td>105</td>
<td>111</td>
</tr>
<tr>
<td>E. C.</td>
<td>30</td>
<td>89</td>
<td>0.74</td>
<td>118</td>
<td>117</td>
</tr>
<tr>
<td>N. F.</td>
<td>44</td>
<td>114</td>
<td>0.65</td>
<td>182</td>
<td>187</td>
</tr>
<tr>
<td>M. F.</td>
<td>67</td>
<td>138</td>
<td>0.51</td>
<td>310</td>
<td>325</td>
</tr>
</tbody>
</table>

**Experimental Confirmation of Equation 11**—The phosphohexose isomerase activity of each of a number of sera was determined at two concentrations, 0.01 and 0.04 cc., per cc. of the reaction mixture. At the lower of these concentrations the amount of substrate changed was frequently within the zero order portion of the reaction, whereas the amount changed at 0.04 cc. was well beyond it. A few representative results are presented in Table II. The reaction is of zero order for substrate changes up to about 30 $\gamma$ of fructose formed as fructose-6-phosphate per cc. of the reaction mixture. At substrate changes greater than this, the time-change function departed from zero order, and the ratio of the change at 0.04 cc. to 4 times that at 0.01 cc. decreased below 1.00. The ratio became progressively lower, the more active the serum, and hence the greater the changes in the substrate.

In contrast, the ratio of the activities of a serum at these two concentrations, expressed in accordance with Equation 11, was, within experimental error, 1.00, no matter at what stage of the reaction the change in
the substrate was measured. The use and confirmation of Equation 11 are in agreement with the general principle that enzyme activity is directly proportional to enzyme concentration when a proper measure of reaction velocity is employed (1, 3, 7).

**DISCUSSION**

Three methods have been available for measuring and expressing the activity of an enzyme: (a) a reaction parameter which characterizes the entire course of the enzyme reaction; (b) the reciprocal of the time necessary to effect a given change in the substrate; (c) the amount of substrate changed in a stated time during the zero order portion of the reaction (1–3). The present paper submits a new method, the use of an equation by means of which the enzyme activity can be expressed in terms of the amount of substrate changed at any stage of the reaction. The relationship implicit in the formulation of this equation, namely the identity of the form of the time-change function with variation in concentration or source of enzyme, should be verified for any particular enzyme to which the proposed method is to be applied. This relationship has been shown in this work to hold for the phosphohexose isomerase activities of various sera. It has previously been demonstrated for other enzyme preparations such as invertase (8), urease (9), and various tissue phosphatases (1).

**SUMMARY**

An equation has been developed by means of which enzyme activity may be expressed in terms of the amount of substrate changed at any stage within or beyond the zero order portion of the reaction. This formulation is illustrated and confirmed by the comparison of the phosphohexose isomerase activities of various sera, and its applicability in the comparison of the activities of other enzymes in biological fluids, tissue extracts, or under varying reaction conditions is indicated. This formulation also permits the use of low initial concentrations of substrates, an advantage when such materials are expensive or scarce.

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MEASUREMENT OF ENZYME ACTIVITY AT ANY STAGE OF A REACTION
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