actual difference between the rates of hydrolysis of glucose-6-phosphate in the diabetic and normal liver decreases progressively as the substrate concentration diminishes. For example, from the average K_s and V_m values given in Fig. 1, it can be calculated that at a glucose-6-phosphate concentration of 10 m*M* the rate of hydrolysis per milligram of tissue is about 75 percent more rapid in the diabetic liver than in the normal liver, whereas, at a substrate concentration of 1 m*M* the difference is about 25 percent. It is obvious that any extension of differences in glucose-6-phosphatase activities observed in vitro to an explanation of the altered carbohydrate metabolism of the diabetic liver must be based upon an actual difference in the rate of hydrolysis of glucose-6-phosphate in vivo rather than upon a difference in potential capacity.

It will be of interest to discover whether the increased K_s values observed with the enzyme from diabetic animals represents an intrinsic difference in property which is retained in the purified enzyme or is a result of an altered milieu in the diabetic liver.

Note added in proof. In recent experiments it has been found that the high K_s values of the enzyme from diabetic animals was restored to the normal level after 48 hours of insulin treatment, and the V_m values were also markedly decreased (*11*).

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6. This investigation was supported by a research grant (A-875, C-2) from the National Institutes of Health, U.S. Public Health Service.
7. Values of 2 m*M* for the K_s of the glucose-6-phosphatase of the microsome fraction of rat liver [R. K. Crane, *Biochim. et Biophys. Acta* 17, 443 (1955)], and of 2.5 m*M* for the K_s of partially purified glucose-6-phosphatase from dog liver (*4*) have been reported. We have obtained an average value of 3.2 m*M* for the enzyme in homogenates of eight normal dog livers.
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10. Langdon and Weakley (*4*) have reported a *competitive* inhibition by glucose for the partially purified enzyme from dog liver, with a K_i of 0.029*M*.
11. A paper describing the details of these experiments is in preparation.

25 February 1958

Method for Adapting the Virus of Rinderpest to Rabbits

The difficulty of adapting the virus of rinderpest to rabbits by serial passage is well known. Baker (*1*) succeeded in adapting the rinderpest virus to rabbits by alternating the inoculations between calves and rabbits. The calf served to maintain sufficient virus in the inocula to overcome the threshold of infection in the rabbits. After a number of alternations sufficient adapted virus was produced to assure the maintenance of serial passage infections.

Shope *et al.* (*2*) have shown that the minimal infective dose of rinderpest virus for the chorioallantoic membrane is roughly in agreement with the subcutaneous minimal infective dose of virus for the calf. It was, therefore, thought that the chorioallantoic membrane might be substituted for the calf in the alternate passing referred to above. With this in mind an experiment was conducted in which a virulent strain of rinderpest virus was successfully adapted to the rabbit. Although this method has no particular advantage other than economic over that evolved by Baker, it is possible that it might be of value in the study of other viruses.

The Kabete strain of rinderpest virus was propagated on the chorioallantoic membrane through 45 serial transfers after the manner described by Shope *et al.* Calf inoculations of earlier passage membranes usually resulted in death on the 8th, 9th, or 10th day. Chorioallantoic membranes from the 42nd passage eggs were inoculated into a calf, and there was no evidence of attenuation of the virus.

Rabbits of various breeds were employed. Only healthy, mature subjects whose normal temperature fell in the range of 102 to 103.5°F were selected for the experiment. The fertile eggs employed were from a healthy flock of mixed breeds.

The inoculum of chorioallantoic membranes for each rabbit consisted of 2.0 ml of a suspension of three chorioallantoic membranes comminuted in 3.0 ml of allantoic fluid. The rabbit spleen suspensions were prepared by grinding each rabbit spleen in 9.0 ml of normal saline. The amount of this suspension deposited on each chorioallantoic membrane was 0.3 ml. None of the tissue suspensions were centrifuged, and the rabbit inoculations (all intravenous) were made slowly in order to avert shock. The 10-day embryonated eggs were incubated for 4 days, and all rabbits whose spleens were employed for inocula were electrocuted on the fourth day after injection.

1) The series of inoculations was begun by inoculating 43rd passage chorioallantoic membranes into rabbit No. 1. The highest temperature recorded was 104.8°F. The spleen from rabbit No. 1 was inoculated on to the first group of chorioallantoic membranes.

2) The first group of membranes was inoculated into rabbits No. 2 and 3. The peak temperature of the former was 103.9°, and that of the latter 104.1°. The spleen from rabbit No. 3 was inoculated on to the second group of chorioallantoic membranes.

3) The second group of membranes was inoculated into rabbits No. 4 and 5. Rabbit No. 4 died as a result of shock. Rabbit No. 5 had a peak temperature of 104.6°. The spleen was inoculated on to the third group of chorioallantoic membranes.

4) The third group of membranes was inoculated into rabbit No. 6. The highest temperature was 104.0°. The spleen was inoculated on to the fourth group of chorioallantoic membranes.

5) The fourth group of membranes was inoculated into rabbit No. 7.

The inoculations were then continued serially in rabbits. The spleen from rabbit No. 7 was inoculated into rabbits No. 8 and 9. The highest temperatures were 103.4° and 104.1° respectively.

The spleen from rabbit No. 9 was inoculated into rabbit No. 10. The highest temperature recorded was 106.2°. The spleen from this rabbit was inoculated into rabbits No. 11 and 12. The post-injection temperatures are listed in Table 1.

The spleen of rabbit No. 12 was removed and each of two calves was given 1.0 ml of a 1:10 dilution in saline.

Calf No. 1, which had been previously immunized against rinderpest, showed no evidence of the disease.

Calf No. 2, which had not been immunized, succumbed to characteristic rinderpest in 12 days. The first high temperature was recorded on the third day following injection, indicating that there was considerable amount of virus present in the spleen of rabbit No. 12.

The temperature curves of rabbits 10, 11, and 12 for the 96-hour postinjection period conformed in general with a typical curve drawn by Baker. These rabbits also exhibited substantially the same signs of illness and gross pathology as Baker observed in his rabbits of comparable passages, except that rabbits 11 and 12, but not rabbit 10, displayed a considerable number of small white foci in the swollen Peyer's patches, sacculus rotundus, tonsilla cecalis major, and appendix. The number of white foci was not as great in either rabbit as is usually observed in rabbits injected with the Nakamura lapinized strain.

Although 43rd-passage membrane virus was employed to initiate the alter-

Table 1. Temperatures recorded in rabbits inoculated with rinderpest virus.

Time after injection (hr)	Temperature (°F)	
	Rabbit 11	Rabbit 12
24	102.5	102.8
32	103.3	104.5
48	105.6	106.5
56	106.0	107.0
72	102.8	103.9
80	103.0	103.2
96	102.4	103.1

nate transfers, it seems likely that earlier passage membranes could be used with equal success. Titrations of fourth- and ninth-passage membranes have shown the virus to be present in considerable amounts (*2*). No doubt, if a sufficient number of serial passages were carried out in the rabbit, the virus would eventually become attenuated for the calf (*3*).

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6 March 1958

Enzymatic Synthesis of Adenyl-oxyluciferin

In the activation of amino acids, acetic acid, and firefly luciferin by adenosine triphosphate (ATP), pyrophosphate and the corresponding acyl-adenylates are presumably formed (*1*). The synthetic adenylates in all three cases are biologically active. Unfortunately, the enzymatic synthesis of these acyl-adenylates has not been demonstrated. Presumably this is due to the tight binding of the adenylates to the enzyme. Recently we have been able to measure the equilibrium constant for the association of adenyl-oxyluciferin (L-AMP) and firefly luciferase. As expected, it was found that the equilibrium constant of the reaction

$$\text{L-AMP} + \text{E} \rightleftarrows \text{E-L-AMP}$$

was approximately 2×10^9. Apparently a similar tight binding with enzyme exists with regard to the amino acid and acetic acid adenylates. If this is true, it is not surprising that it has been difficult to demonstrate the enzymatic formation of the adenylates of these compounds. However, the intense fluorescence of oxyluciferin makes it possible to measure the disappearance of quantities which are less than the amount of enzyme added. Because of this sensitivity of the fluorescence technique, we have been able to measure the enzymatic formation of L-AMP in the following reaction:

$$\text{ATP} + \text{L} + \text{E} \rightleftarrows \text{E-L-AMP} + \text{POP} \quad (1)$$

The measurement of L-AMP synthesis depends on the following differences in properties between chemically synthesized L-AMP and oxyluciferin: (i) L-AMP has a fluorescence intensity which is only 2 percent of the intensity of oxyluciferin. (ii) At a *p*H of 3 oxyluciferin is extractable into ethyl acetate, whereas L-AMP is not. The solubility of the oxyluciferin in ethyl acetate depends upon the dissociation of the carboxyl group. (iii) When paper chromatography and the solvent system described in Table 1 are used, L-AMP has an R_f of 0.55, while oxyluciferin has an R_f of 0.40.

The results of an experiment in which oxyluciferin, luciferase, $MgSO_4$ and ATP were incubated at *p*H 7.1 are shown in Table 1. The reaction was initiated with ATP. Following the addition of ATP there was an immediate decrease in fluorescence corresponding to the disappearance of oxyluciferin approximately equal to the amount of luciferase present. When the reaction mixture was acidified and extracted with ethyl acetate, the excess oxyluciferin was removed from the reaction mixture. The nonextractable, weakly fluorescent material in the aqueous phase had an R_f of 0.55 which is identical to that of synthetic adenyloxyluciferin. Alkaline hydrolysis gives an increase in the fluorescent intensity and a product with an R_f of 0.4. This, and other evidence which has been recently published, indicates clearly that the material in the aqueous phase is adenyl-oxyluciferin (*2*).

By fluorescence measurements, it has been possible to determine equilibrium concentration of oxyluciferin from which the equilibrium constant for reaction 1 can be calculated. It should be noted that in this reaction the enzyme must be considered in the equilibrium determination. Measurements made on the forward and reverse reactions both give a value of K approximately equal to 2.0×10^5. By using this constant, along with the dissociation constant for luciferase-L-AMP complex, the free energy of hydrolysis of L-AMP can be calculated. If it is assumed that the free energy of hydrolysis of the second phosphate bond in ATP is the same as for the terminal phosphate bond (*3*), the free energy of hydrolysis is obtained by adding the following reactions:

Table 1. Results. The reaction mixture contained the following: oxyluciferin, 3.2 mμmole; $MgSO_4$, 10 μmole; luciferase, 2.0 mμmole; ATP, 4 μmole; brought to final volume of 2.0 ml with 0.1*M* tris-maleate buffer, *p*H 7.1.

Reaction mixture	Fluorescence* intensity	R_f
− ATP	80	0.40
Complete	30	0.55†

* Arbitrary units; excitation, 360 mμ; emission, 540 mμ.
† After ethyl acetate extraction; paper Whatman No. 3-MM; solvent, 30:70 (by volume) mixture of 1*M* ammonium acetate and 95 percent ethanol, *p*H 7.5.

	$\Delta F°'$(kcal)
$\text{ATP} + \text{L} + \text{E} \rightleftarrows \text{E-L-AMP} + \text{POP}$	− 7.2
$\text{E-L-AMP} \rightleftarrows \text{E} + \text{L-AMP}$	+ 12.6
net $\text{ATP} + \text{L} \rightleftarrows \text{L-AMP} + \text{POP}$	+ 5.4
$\text{POP} + \text{AMP} \rightleftarrows \text{ATP} + \text{H}_2\text{O}$	+ 7.7
net $\text{L} + \text{AMP} \rightleftarrows \text{L-AMP} + \text{H}_2\text{O}$	+ 13.1

These calculations were made on the assumption that Mg^{++} has no appreciable effect on the equilibrium of the activation reaction. This point is now being investigated. It is not surprising that the free energy of hydrolysis of L-AMP is considerably higher than that of ATP, since coenzyme A reacts readily with L-AMP to produce AMP and oxyluciferyl-coenzyme A.

The biological significance of the tight binding of acyl adenylates to the enzyme is not clear. However, it should be pointed out that the present evidence indicates that such tight binding restricts the reactivity of the adenylates. For example, L-AMP is capable of reacting with SH compounds such as coenzyme A and cysteine to form the corresponding oxyluciferin derivatives and adenylic acid. However, cysteine is capable of reacting only nonenzymatically with L-AMP, whereas coenzyme A can react with L-AMP only when it is bound to luciferase (*4*).

Note added in proof: M. Karasek *et al.* have recently demonstrated the enzymatic synthesis of tryptophan-AMP (*5*).

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