tary replication, we have to confront the double difficulty of unwinding and rewinding. The present mechanism is no exception; the parent DNA unwinds exactly N_0 turns, and the daughters rewind exactly N_0 turns, N_0 being the winding number of the DNA molecule. However, the difficulty is reduced to a minimum by rewinding immediately after unwinding so that there is no large number of unwound turns to cause difficulty both in unwinding and rewinding. In the previous paper, it is stated that the unwinding angular velocity $\bar{\omega}(t)$ is a monotonically decreasing function of time or of the number of turns unwound. In the present mechanism, the unwound turns are removed by the rewinding of the daughters so that the unwinding is operated at all times near the starting point of the $\bar{\omega}(t)$ curve, and the average value of $\bar{\omega}(t)$ is very close to its maximum value $\bar{\omega}(0)$. Thus, the unwinding time in the present mechanism is very close to the lower limit T_l in the previous paper. Unwinding *in vivo* is therefore much faster than *in vitro*.

The author wishes to thank Professor Philip Morrison for many discussions and criticisms on the manuscript.

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[7] It is likely that the daughters are more disposed to rotate in the direction of the arrows than backward, since the backward rotation may strain the bonding angles of the chains in the daughter helices in the neighborhood of P and Q. Such a bias would speed up the random process.

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*THE EFFECTS OF pH AND TEMPERATURE ON THE KINETICS OF THE PHOSPHORYLASE REACTION**

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The dependence of muscle phosphorylase on 5′-AMP[1] for activity, absolute in the case of phosphorylase *b* and relative in the case of phosphorylase *a*, has been explained by an effect of 5′-AMP on the affinity of the enzyme for its substrates, inorganic P and glycogen.[2] Each of these substrates in turn influences the affinity of the enzyme for 5′-AMP. Thus, there is a reciprocal interaction between the binding sites for substrates and cofactor, suggestive of a conformational alteration of the protein.

Two recent papers on this subject have appeared.[3, 4] Madsen[3] reported results similar to those described above for the interaction of glucose-1-P and 5′-AMP with phosphorylase *b*. He found that increasing the concentration of glucose-1-P results in an increase in the affinity for 5′-AMP and vice versa. Lowry *et al.*[4] found that 5′-AMP decreased the dissociation constant of each of the substrates of phosphorylase *a*. Madsen[3] also studied the interaction between ATP, a competitive inhibitor[5] of 5′-

AMP and glucose-1-P. At a fixed concentration of ATP the saturation curve for glucose-1-P had a sigmoidal shape, whereas in the absence of inhibitor the usual hyperbolic curve was obtained.[6] It is of interest that the same kinetics were described for the inhibition of phosphorylase *a* by glucose and that 5'-AMP counteracted this inhibition.[7]

The results on the effect of pH and temperature reported in this paper are in accord with previous observations that muscle phosphorylase belongs to a class of proteins of multiple subunit structure which can easily be induced to undergo conformational alteration in response to variations in experimental conditions.

Experimental Procedure.—In one group of experiments arsenate replaced phosphate, and its concentration was varied while glycogen, the second substrate, was present at saturating concentrations. The advantage of using arsenate instead of phosphate is that under these conditions the reaction proceeds linearly with time at all concentrations of arsenate. The K_m for arsenate is of the same order as that of phosphate.[2] The product of the reaction, as Katz and Hassid[8] have shown, is free glucose, presumably because the glucose-1-arsenate which would be formed as an intermediate is unstable and decomposes spontaneously. No evidence could be obtained that this decomposition became rate-limiting as a result of changes in pH or temperature. In another series of experiments the concentration of glycogen was varied, while glucose-1-P was present at saturating concentrations.

The same preparation of phosphorylase *a*, kindly supplied by Dr. Illingworth, was used in all experiments. The enzyme had been recrystallized 4 times, the last time after treatment with acid-washed Norit A so as to free it of any possible contamination with nucleotides. The glycogen was also treated with Norit as described previously[2] in order to free it of nucleotides. The buffer used in the experiments with arsenate at different pH contained 0.025 *M* Na-glycero-P, 0.025 *M* Tris-acetate, 0.0025 *M* EDTA-Na_2, and 0.0012 *M* 2-mercaptoethanol, adjusted to the desired pH with acetate. When temperature was the variable, Tris-acetate was omitted, Na-glycero-P was 0.05 *M*, and the pH was 6.75. A typical reaction mixture contained 0.4 ml of buffer, 0.1 ml of 1% egg albumin, 0.2 ml of 1% glycogen, 0.1 ml of 0.01 *M* 5'-AMP solution or 0.1 ml of water, and 0.1 ml of a solution containing varying concentrations of arsenate. The reaction was started by addition of 0.1 ml of enzyme solution containing about 50 μg of protein. Incubation was usually for 15 min at 31° unless stated otherwise. The reaction was terminated by the addition of perchloric acid, followed by neutralization with K_2CO_3 and centrifugation in the cold. An aliquot of the supernatant fluid was then analyzed for glucose by adding ATP, Mg acetate, crystalline yeast hexokinase, and glucose-6-P dehydrogenase. After taking an initial reading, the reaction was started by adding TPN to the reference and experimental cuvettes. The formation of TPNH was followed at 340 mμ in a Zeiss PMQ II spectrophotometer until an endpoint was reached. Each series of measurements included a glucose standard. The crystalline enzymes were obtained from Boehringer and Sons. Glucose-6-P dehydrogenase was freed of excess $(NH_4)_2SO_4$ by dialysis as previously described.[2]

Glucose-1-P was used as substrate at a concentration of 0.045 *M* which is nearly 10 times its K_m value.[9] In most experiments, less than 15% of the substrate reacted during the incubation period and, hence, the reaction rate, calculated from the liberation of inorganic P, remained linear with time. When pH was the variable, the glucose-1-P solution, adjusted to the desired pH, served as the buffer. The reaction was started by adding about 20 μg of phosphorylase *a* to 0.2 ml of reaction mixture in a Klett colorimeter tube. Incubation was for 10 min at 31° unless stated otherwise. Since no deproteinization was required, the colorimetric determination of the inorganic P by the Fiske and Subba-Row method could be carried out in the same tube which was used for incubation. When 5'-AMP was added, its concentration was 1×10^{-3} *M*. Further details are given in the tables and graphs.

Results.—Effect of pH: Table 1 shows that the addition of 5'-AMP decreased the K_m (increased the affinity) of arsenate for phosphorylase *a* by a factor of about 5 at all pH values. Increasing the pH from 5.5 to 8.0 resulted in a threefold decrease of K_m for arsenate. This change in K_m occurred both in the presence and

in the absence of 5'-AMP. On plotting K_m versus pH, an inflection in the curve occurred around pH 6.7. It is in this region that arsenate has its pK_2. In contrast to arsenate, the K_m value for glycogen, a nonionizable substrate, showed little change over the pH range from 5.5 to 7.3 (Table 1). However, different binding sites are involved in these two cases. Without additional information one cannot decide whether the pH effect on K_m of arsenate reflects a change in the ionization of the substrate, of some group on the enzyme involved in the binding of arsenate, or of both.

TABLE 1

K_m AND V_{max} FOR ARSENATE AND FOR GLYCOGEN AT DIFFERENT pH VALUES

	Arsenate				Glycogen	
	K_m ($M \times 10^{-3}$)		V_{max}*		K_m ($M \times 10^{-5}$)†	V_{max}‡
pH	−5'-AMP	+5'-AMP	−5'-AMP	+5'-AMP	+5'-AMP	+5'-AMP
5.5	42	8	0.53	0.9	13	6.3
6.0	43	9	1.10	1.10		
6.3					15	8.8
6.75	23	3	0.77	0.84	10	7.3
7.3					10	6.1
7.5	21	4	0.34	0.49		
8.0	16	3	0.13	0.24		

* μmoles Glucose formed during 15 min of incubation at 31° with 50 μg phosphorylase *a* and 2 mg of glycogen per ml.
† Concentration in terms of moles of terminal glucose units at the nonreducing end of the chains.
‡ μmoles Inorganic P formed per ml from 0.045 *M* glucose-1-P during 10 min of incubation at 31° with 23 μg of phosphorylase *a*.

In Table 1, the V_{max} values for arsenate at intermediate pH values from 6.0 to 7.5 were nearly the same with and without 5'-AMP, despite a fivefold difference in the K_m values in the presence and absence of the cofactor. This was also the case in previous experiments with phosphorylase *a* in which the formation of glucose-1-P from glycogen and inorganic P was measured at pH 7.5 in the presence of Mg^{++} with the aid of a coupled enzyme system.[2] Similar results, that is, large changes in K_m but no change in V_{max}, were also found in a series of experiments with phosphorylase *b*.[2] The results in Table 1 show, however, that this is not an invariable characteristic of the catalytic system. At the extremes of the pH range, V_{max} without 5'-AMP was only about $1/2$ of that with 5'-AMP. As will be shown subsequently, differences in V_{max} in the presence and in the absence of 5'-AMP were also observed at low temperature.

According to Table 1, the pH optimum of the enzyme, based on the V_{max} values for arsenate, was around pH 6.0 with and without 5'-AMP. Additional data on the effect of pH on the rate of the reaction at high concentrations of arsenate or glucose-1-P are shown in Figure 1. The pH optimum with glucose-1-P was in the same range as that of arsenate. It should be emphasized that in view of the large change in the K_m value with pH, the pH optimum of the phosphorylase reaction will depend on the substrate concentration used for its determination. For example, it can be calculated from the data in Table 1 that at an arsenate concentration of 0.01 *M* the optimum would be shifted from pH 6.0 to pH 6.75 both in the presence and absence of 5'-AMP. In previous experiments[7] with 0.016 *M* glucose-1-P as substrate, the pH optimum was found to be close to 6.7. It can be seen that the descending limb of the curve in Figure 1 has an inflection at about pH 7.2. By plotting the same data as log *v* versus pH one obtains an extrapolated pK value at 6.8 which is characteristic for many enzymes.

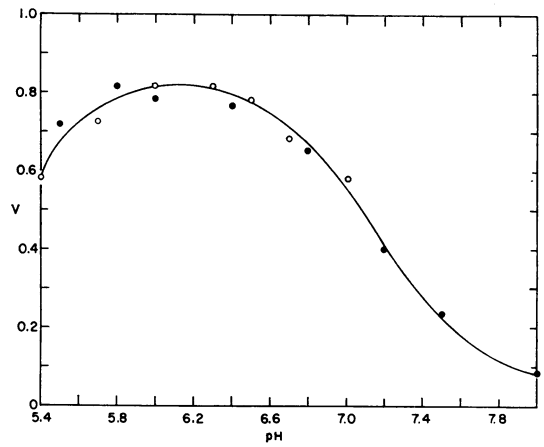

FIG. 1.—Effect of pH on the rate (v) in the presence of 5′-AMP. (●) Experiments with 0.1 M arsenate and 0.2% glycogen. (○) Experiments with 0.045 M glucose-1-P and 0.5% glycogen. The rates given for the two substrates are relative, such that the highest rate observed with glucose-1-P was set equal to the highest rate observed with arsenate.

Effect of Temperature.—The data in Table 2 show that temperature is another variable which profoundly influences the K_m values for substrates. Thus, the K_m value for arsenate in the absence of 5′-AMP increased 10-fold over the temperature interval from 18° to 38°. With 5′-AMP there was a twofold increase in the K_m of arsenate and a fivefold increase in the K_m of glycogen over this temperature range.

TABLE 2

K_m AND V_{max} FOR ARSENATE AND FOR GLYCOGEN AT DIFFERENT TEMPERATURES AT pH 6.75

Temperature, °C	Arsenate				Glycogen	
	K_m ($M \times 10^{-3}$)		V_{max}*		K_m ($M \times 10^{-6}$)*	V_{max}*
	−5′-AMP	+5′-AMP	−5′-AMP	+5′-AMP	+5′-AMP	+5′-AMP
12.5					4	0.84
18.0	5	6	0.06	0.016		
23.5					5	3.4
28	11	5	0.33	0.64		
31	23	3	0.77	0.84	10	7.3
38	51	11	1.18	1.18	21	10.4
41					28	9.0

* As defined in Table 1, except for the difference in temperature.

From previous measurements[10] with 0.016 M glucose-1-P, it was known that the ratio of activity (−AMP/+AMP) changed from 0.32 at 0° to 0.65 at 30°. As can be seen from the V_{max} values for arsenate in Table 2, the present results are similar. The activity ratio changed from 0.4 at 18° to 0.9 at 31°, and to 1.0 at 38°. The meaning of this change in the ratio with temperature has been pointed out.[10] The energy of activation of the catalytic system, as calculated from the slopes in an Arrhenius plot, is smaller when phosphorylase a is combined with 5′-AMP than when the enzyme is acting in its absence. The temperature range previously covered was from 0° to 30° and the μ value in the presence of 5′-AMP was 20.8 Kcal. The μ value in the absence of 5′-AMP (24.6 Kcal) was probably too low because owing to an increase in K_m the substrate concentration used (0.016 M glucose-1-P) would have been insufficient to keep the enzyme saturated with in-

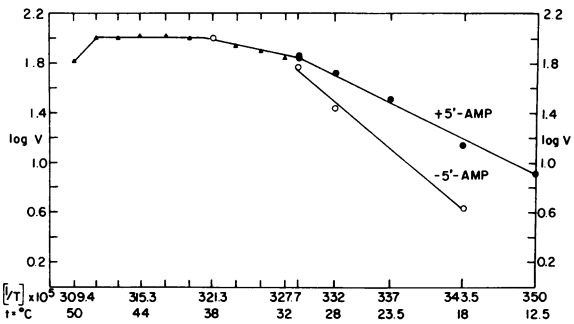

FIG. 2.—A plot of log v against $1/T°K$. The data were plotted relative to the rates observed in each case at 38° which was set equal to 100. (●) V_{max} values with 5'-AMP in Table 2. (○) V_{max} values without 5'-AMP in Table 2. (▲) Separate experiments with 5'-AMP, 0.2% glycogen and 0.2 M arsenate; the time of incubation was 7.5 min.

creasing temperature. In Figure 2, the values for V_{max} in Table 2 have been plotted, and additional data have been obtained at saturating substrate concentrations and in the presence of 5'-AMP for the temperature range from 31° to 50°. Up to 31° the results confirm those previously reported.[10] The μ value for the experiments with 5'-AMP was 20.2 Kcal and that without 5'-AMP, about 30 Kcal.

Special interest attaches to the curve in Figure 2 above 31°. A new slope is established between 31° and 38° with a μ value of about 10 Kcal. A further rise in temperature up to 48° has practically no effect on the rate and it is only above this temperature that the rate begins to fall. At 50° the rate was still 80 per cent of that at 40°. (In the absence of 5'-AMP, the rate at 49° was only 9% of that observed at 39°.[7]) One possible interpretation of the change in the slope in an Arrhenius plot is that within a critical temperature range the catalyst assumes a different conformational state. This might lead to a change in the rate-limiting step of the over-all reaction. In line with such an assumption is that in the present case there is also a basic change in the kinetics of the enzyme within this critical temperature range. This is illustrated in Figure 3, where it is shown that in a Line-

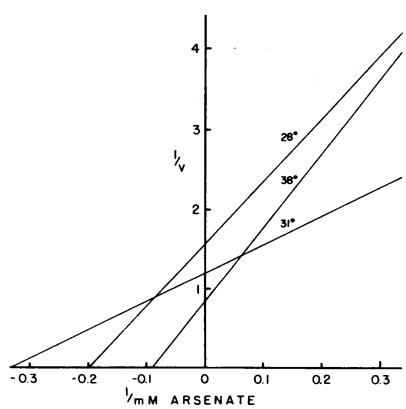

FIG. 3.—Lineweaver-Burk plots based on the values for K_m and V_{max} of arsenate in the presence of 5'-AMP shown in Table 2. The range of concentrations of arsenate corresponds to that actually used in the K_m determinations.

weaver-Burk plot the line representing the experiment at 38° intersects the line representing the experiment at 31° at a point to the right of the ordinate. On the other hand, the lines which represent the experiments at 28° and 31° intersect at a point to the left of the ordinate. Similar results are obtained when the data for glycogen at these temperatures are plotted. In general, the substrate concentration at which the lines cross is given by the expression: $1/S = V_1 - V_2/(K_{m1}V_2 - K_{m2}V_1)$. Applying this equation to the data in Table 2, $1/S$ gives a negative value for pairs at different temperatures up to 31°. In these cases the lines cross to the left of the ordinate. Positive values are obtained for $1/S$ if one of the pairs is from an experiment at a temperature above 31°. In these cases the lines cross to the right of the ordinate. A consequence of this kinetic behavior is that at high substrate concentration the rate of the reaction is greater at 38° than at 31°, whereas the opposite is true at low substrate concentration. Similar observations have been made by Lowry *et al.*[4] These authors have also shown that the temperature effects observed at low substrate concentration are reversible, that is, if after incubation at 38°, the temperature was decreased to 28°, the rate increased. Reversibility would be expected if a conformational change were involved in the temperature effect. In terms of the kinetic parameters affected it is interesting to note that up to 31° V_{max} is more strongly influenced than K_m, whereas above 31° the effect of a rise in temperature is mainly on K_m. The temperature effects on the rate of the reaction at high and low substrate concentration are thus entirely predictable by the changes in these parameters. It will also be understood why the temperature optimum of the phosphorylase *a* reaction varies with the substrate concentration. At levels of inorganic P and glycogen considerably below half saturation of the enzyme, Lowry *et al.*[4] reported a temperature optimum of 23° when 5′-AMP was present and of 18° in its absence. On the other hand, a temperature optimum of 38° is found at saturating substrate concentrations (Table 2, Fig. 2).

Discussion.—Changeux, Ullman, and Monod[11] have recently proposed that in analogy to hemoglobin, all "allosteric" proteins consist of identical subunits which are held together by noncovalent bonds. A new equilibrium between various conformational states of an enzyme would then be attained through modifications of subunit interaction. Muscle phosphorylase is unusual in that it seems to be capable of undergoing several "allosteric" transitions. One of these results from the interaction with 5′-AMP and is characterized by large changes in the K_m of the substrate binding sites with little change in V_{max}. With increasing temperature there is a transition from an effect principally on V_{max} to one principally on K_m, whereas with increasing pH the transitional state seems to be less sharply defined since there is a simultaneous effect on both parameters. The reason for these varying effects could be that the quaternary structure of phosphorylase is complex. First, there is the interaction of the 2 subunits in phosphorylase *b* and the corresponding interaction of the phosphorylated subunits in phosphorylase *a*. This interaction differs considerably from that involved in the dimerization of 2 phosphorylated *b* units to form the phosphorylase *a* tetramer. For example, high salt concentration results in the dissociation of phosphorylase *a* into two subunits which are reported to have higher specific activity than the undissociated phosphorylase *a*.[12] Relevant to this discussion is the observation by Levy *et al.*[13] that whereas the ATPase activity of myosin alone showed normal temperature behavior in an

Arrhenius plot, that of actomyosin showed 2 slopes corresponding to activation energies of 25 Kcal below 16° and 12 Kcal at temperatures above 16°. Apart from the difference in the temperature of transition, the Arrhenius plots for actomyosin and phosphorylase *a* are quite similar.

A study of the kinetic behavior of the catalytic system permits one to define experimental conditions under which transitions indicative of a structural alteration occur. It seems clear, however, that deeper insight into the structural changes which enzymes can undergo under different environmental conditions requires a different experimental approach as yet not fully available. Equally difficult is an experimental approach to the question of the extent to which these structural modifications play a role *in vivo*.

Summary.—Arsenate was used in place of phosphate in the phosphorylase *a* reaction because kinetic measurements were thereby simplified. The effect of an increase in pH from 5.5 to 8.0 was a decrease in the K_m of arsenate by a factor of about 3, both in the presence and absence of 5′-AMP. Owing to this change in K_m, the pH optimum was dependent on the substrate concentration. The effect of addition of 5′-AMP was a decrease in the K_m of arsenate by a factor of about 5 at all pH values. At intermediate pH values the V_{max} was nearly the same with and without 5′-AMP, but became lower without 5′-AMP at the extremes of the pH range. Large changes in the K_m for arsenate and for glycogen were produced by an increase in temperature from 12° to 38° at constant pH. For this reason the temperature optimum of the phosphorylase reaction was also dependent on the substrate concentration. In an Arrhenius plot of the reaction rates obtained at saturating substrate concentrations up to a temperature of 31°, the energy of activation was lower in the presence of 5′-AMP than in its absence. Between 31° and 38°, a new slope was established with and without 5′-AMP. In this temperature range there was likewise a change in the kinetics of the system such that the rate of the reaction was greater at 38° than at 31° at high substrate concentration, whereas the opposite was true at low substrate concentration. In the presence of 5′-AMP no decrease in the rate of the reaction occurred up to 48°. At 50° the rate was still 80 per cent of maximum. In the absence of 5′-AMP the rate declined much more rapidly with increasing temperature. These observations suggest that phosphorylase can undergo reversible changes in its quarternary structure, presumably as the result of altered subunit interaction.

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[1] Abbreviations are as follows: 5′-AMP, 5′-adenosine monophosphate; inorganic P, orthophosphate; Tris, Tris (hydroxymethyl) aminomethane; EDTA, ethylene-diamine tetraacetate. K_m, apparent Michaelis constant, and V_{max}, extrapolated maximal velocity in a Lineweaver-Burk plot.

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[5] Parmeggiani, A., and H. E. Morgan, *Biochem. Biophys. Res. Commun.*, **9**, 252 (1962). As shown by these authors, phosphorylase *a* was not inhibited by ATP when the ratio ATP/5′-AMP was 13, whereas phosphorylase *b* was inhibited about 50 per cent. It was found recently that when the above ratio was about 10,000, phosphorylase *a* was inhibited about 40 per cent. It is thus clear that the two forms of phosphorylase differ markedly in their sensitivity to ATP inhibition.

[6] Madsen (private communication) found that Mg ions lower the K_m for 5'-AMP without affecting the V_{max}. Owing to this effect, the inhibition by ATP is markedly diminished in the presence of this metal ion. Similarly, the effect of 5'-AMP on the interaction of phosphorylase with its substrates could be modified by the presence of Mg ions and result in different numerical values for kinetic parameters. In previous papers[2, 4] an auxiliary enzyme system was used to measure the rate of the phosphorylase reaction and this necessitated the addition of Mg ions. It should therefore be emphasized that the present experiments were conducted in the absence of Mg ions.

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[9] The K_m of glucose-1-P for phosphorylase *a* has been redetermined at saturating concentrations of glycogen and in the presence of 1×10^{-3} *M* 5'-AMP. At pH 6.8 at 31° in 0.04 *M* Na-citrate-0.004 *M* 2-mercaptoethanol buffer a value of 4.8×10^{-3} *M* was found. Madsen[3] reported a value of 5.1×10^{-3} *M* for phosphorylase *b* at pH 6.7 and 30°.

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[11] We are indebted to Dr. Monod for sending us an unpublished manuscript entitled "Un modèle plausible de la transition allostérique."

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*EXPERIMENTAL PRODUCTION OF TESTICULAR TERATOMAS IN MICE**

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Testicular teratomas are rare in mice except in the inbred strain 129. Two per cent of the males of some sublines of this strain develop spontaneous teratomas congenitally.[1, 2] There is much evidence that these tumors are derived from primordial germ cells,[3] and it is known that the teratocarcinogenetic process is initiated before 15 days and not later than 17 days of gestation.

The incidence of spontaneous testicular teratomas in strain 129 is strongly influenced by environmental and genetic factors.[4] They occur more frequently in the left than the right testis and they are twice as frequent in second and later litters as in first. Susceptibility to teratocarcinogenesis is probably determined by multiple genes, but animals carrying a single gene, Steel (Sl^J), have twice as many tumors as their non-Steel littermates.

Testicular teratomas can be induced in fowl by intratesticular injection of toxic salts and hormones (see Guthrie[5]). Bresler[6] obtained two testicular teratomas in adult mice injected intratesticularly with copper sulphate and testosterone propionate. This observation is noteworthy in view of the fact that all of the teratomas in genetically susceptible strain 129 mice arise prenatally. This means that teratomas in mice may originate from either primordial germ cells (as they do in strain 129) or probably from spermatogonia in adults.

The original aim of this investigation was to find out if teratomas would arise in testes derived from genital ridges transplanted to adult spleens and testes. If so, would the graft site influence the pattern of differentiation of tissues in tera-