suggest a working hypothesis that oncogenesis in this system entails epithelial acquisition of the ability to produce growth-sustaining material(s) normally delivered and regulated by the supporting mesenchyme.

The information that host cells undergo division within the tumors raises the possibility of host cell recruitment into the neoplastic population, as shown by Ponten (*10*) in some RNA virus–tumor systems. The evidence is against this as follows: (i) host cells were euploid in transplant generations as well as in primaries, whereas donor (tumor) cells became more frequently aneuploid in serial transplant; (ii) the proportion of host cell metaphases did not increase progressively during one to four transplant generations; (iii) the histologic structure of the tumors was invariably typical of salivary gland tumors, and salivary gland tissue of the host could not have been present at the transplant sites; (iv) the latent period for appearance of transplants was shorter (as brief as 10 days) than that for tumor induction by virus; and (v) the dividing lymphoid (host) cells in the tumors offer a simpler, more consistent explanation.

Distinguishing between epithelial and connective tissue neoplasms is not usually difficult, but a number of classical problematic examples have perennially stimulated debate among histopathologists. In man, some of the most controversial examples include the mixed tumors of salivary glands (*11*). The procedure used in this study cannot be applied to human neoplasms, but it has yielded strong evidence that certain experimental tumors with predominant connective tissue features actually arise from epithelial cells. From events during embryogenesis it is known that epithelial and mesenchymal modes of cell existence are interconvertible under appropriate conditions (*12*).

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Ammonia Production in Muscle: The Purine Nucleotide Cycle

Abstract. *Experiments are reported which throw new light on the problem of ammonia production by muscle and, probably, by other tissues.*

Muscular work is accompanied by the production of ammonia (*1–8*). The adenylate deaminase (E.C. 3.5.4.6) reaction (*9*) is the major source of ammonia in muscle (*4, 7*) (Eq. 1).

Adenosine monophosphate (AMP) + H_2O → inosine monophosphate (IMP) + NH_3 (1)

The amount of ammonia produced by frog skeletal muscle is proportional to the work done by the muscle (*2, 6*). However, the deamination of AMP is not directly involved in muscular contraction (*10*). The regeneration of AMP from IMP occurs in two steps which are catalyzed by adenylosuccinate synthetase (E.C. 6.3.4.4) (*11–13*) and adenylosuccinase (E.C. 4.3.2.2) (*11, 13*) (Eqs. 2 and 3).

IMP + aspartate + guanosine triphosphate (GTP) → adenylosuccinate + guanosine diphosphate (GDP) + P_i (2)

adenylosuccinate → AMP + fumarate (3)

We report here that extracts of rat skeletal muscle produce ammonia from aspartate in a cyclical reaction sequence under conditions that mimic muscle doing work. The cycle consists of the reactions catalyzed by adenylate deaminase, adenylosuccinate synthetase, and adenylosuccinase. Supporting this conclusion is the finding that ammonia production from aspartate shows a catalytic requirement for either IMP, AMP, or adenylosuccinate. The net effect of the "purine nucleotide cycle" is the formation of fumarate and ammonia from aspartate, and, more indirectly, from glutamate. Possible metabolic functions of the cycle are discussed.

The following comments provide a rationale for the experimental approach which was adopted. Details of the experiments are given in the legends to the figures and table.

Adenylosuccinate, AMP, and IMP have strong absorption spectra. Although they overlap somewhat, these spectra can readily be distinguished from one another (Fig. 1). Changes in the relative amounts of the three nucleotides can be followed by obtaining ultraviolet absorption spectra as a function of time. The amounts of IMP, adenylosuccinate, and AMP present are then calculated from the extinction coefficient of each substance at various wavelengths. The wavelengths used were 281, 270, and 262.5 nm. The calculation of the concentration of each of the three nucleotides is particularly

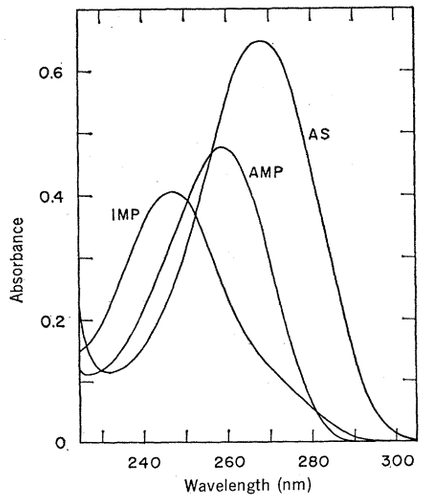

Fig. 1. Absorption spectra of IMP, AMP, and adenylosuccinate (AS). Spectra were obtained on a Cary split-beam, recording spectrophotometer with neutral solutions of 34.8 μM IMP, 32.1 μM AMP, and 34.1 μM adenylosuccinate in a cuvette with a 1-cm light path.

Table 1. Catalytic role of IMP in ammonia production from aspartate. Conditions were those described in Fig. 2. The amount of IMP in the initial reaction mixture was 0.52 μmole/ml. Portions of the complete reaction mixture and the two controls were analyzed for ammonia at the end of each half cycle of the complete reaction mixture. The analyses showed that ammonia was released only during the AMP → IMP half of the cycle.

Omissions from reaction mixture	Ammonia concentration (μmole/ml) at:				
	Start of 1st cycle	End of 1st cycle	End of 2nd cycle	End of 3rd cycle	End of 4th cycle
None	0.04	0.49	0.93	1.26	1.71
IMP	0.04	0.03	0.05	0.06	0.07
Aspartate	0.02	0.01	0.01	0.02	0.03

simple when their total concentration remains constant, as was the case in our experiments.

The reaction sequence IMP → adenylosuccinate → AMP → IMP → and so on, can be demonstrated in particle-free extracts prepared from muscle, provided that certain precautions are taken. First, endogenous substrates must be removed if it is desired to demonstrate the dependence of the reaction sequence on substrates such as purine nucleotides or aspartate. This can be achieved by gel filtration of the extract on a column of Sephadex G-50, or by a short dialysis. Second, a GTP-regenerating system must be provided to counter the inhibitory effects of GDP on the adenylosuccinate synthetase reaction. Third, complete conversion of AMP to ATP must be avoided, or, if it occurs must be reversed; otherwise no AMP will be available for the adenylate deaminase reaction. In theory the second and third conditions are met by providing a specific GTP-regenerating system without providing an ATP-regenerating system. Unfortunately a specific GTP-regenerating system is not readily available. Because of this a relatively unspecific nucleoside triphosphate (NTP)-regenerating system has been employed, consisting of creatine phosphate and endogenous creatine phosphokinase, in conjunction with hexokinase which is specific for converting ATP back to adenosine diphosphate (ADP). Endogenous myokinase then generates an adequate concentration of AMP.

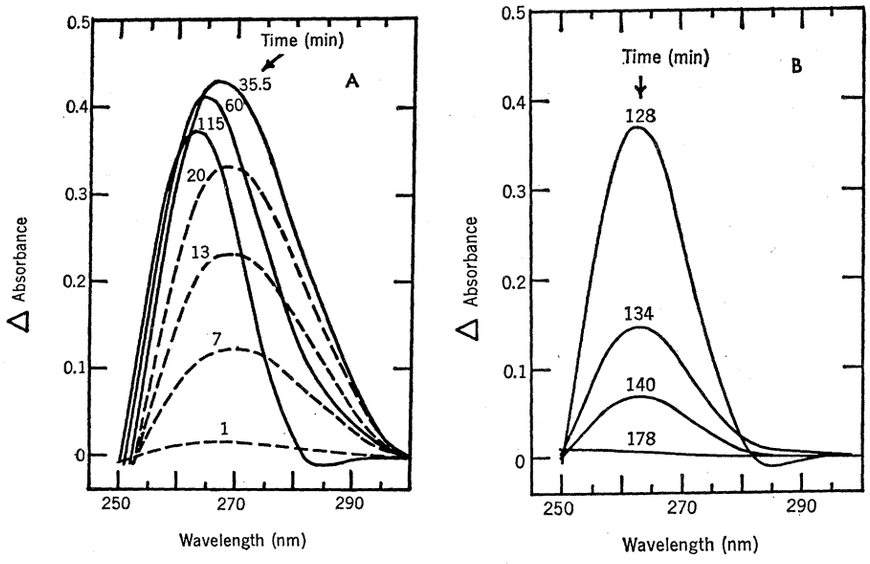

Fig. 2. Conversion of IMP to AMP by way of adenylosuccinate by an extract of leg muscle of rat. (A) The complete reaction mixture contained 0.52 mM IMP, 0.29 mM GTP, 4 mM aspartate, 27 mM imidazole hydrochloride buffer (pH 6.7), 8.3 mM $MgCl_2$, 1.67 mM creatine phosphate, 1.2 units per milliliter of yeast hexokinase (specific activity 70 unit/mg), and rat muscle extract equivalent to 1.0 mg of protein per milliliter. The protein extract contributed 2.5 mM orthophosphate, 47 mM KCl, 0.83 mM ethylenediaminetetraacetate (EDTA), and 17 μM dithiothreitol to the reaction mixture. The reaction was started by adding the protein extract and was run at 31°C. For the spectral scans, a Cary 15 recording spectrophotometer was used with a reference cuvette that contained the same reaction mixture except that it lacked aspartate. The light path was 1 mm. Each scan was started at the time indicated. For the sake of clarity, scans showing the rise in adenylosuccinate concentration are drawn as dashed lines, whereas scans showing the maximum and subsequent decline in adenylosuccinate concentration are drawn in solid lines. The muscle extract was prepared as follows. Rat leg muscle was minced and suspended in three volumes of solution consisting of 90 mM potassium phosphate (pH 6.5), 180 mM KCl. The suspension was blended for 20 seconds in a Waring blendor and stirred in the cold for 1 hour. It was centrifuged first at 31,000g for 10 minutes, and then at 85,000g for 30 minutes. The supernatant from the high speed centrifugation was placed on a column of Sephadex G-50 and eluted with a mixture containing 15 mM potassium phosphate (pH 6.5), 280 mM KCl, 5 mM EDTA, and 0.1 mM dithiothreitol. The pooled peak fractions contained 6.2 mg of protein per milliliter. (B) Conversion of AMP back to IMP. When the formation of AMP described in Fig. 2A had ceased (after 127 minutes), 8.3 μl of 0.2M 2-deoxyglucose per milliliter of reaction mixture was added to give a final concentration of 1.67 mM 2-deoxyglucose. Spectral scans were then continued as described.

An experimental demonstration of the sequential operation of adenylosuccinate synthetase and adenylosuccinase in an extract from rat skeletal muscle is shown in Fig. 2A. Sixteen spectral scans from 310 to 245 nm were run at appropriate intervals after the start of the reaction. Several of these scans are shown in the figure. The concentrations of IMP, adenylosuccinate, and AMP were calculated from these scans (Fig. 3A). The figure shows the appearance of adenylosuccinate and AMP, and the disappearance of IMP. Adenylosuccinate builds up rapidly at first, levels off, and then declines. After an initial lag, AMP accumulates at a steady rate. Much if not all of the AMP formed was probably converted to ATP by the regenerating system, leaving at most very low concentrations of AMP. Very little or none of the AMP formed was deaminated during this phase, as was indicated by ammonia analyses. It should be pointed out that relatively high concentrations of GTP were present throughout, which inhibited adenylate deaminase. Orthophosphate also inhibits the enzyme. The liberation of orthophosphate by the adenylosuccinate synthetase reaction therefore would tend to increase the inhibition of adenylate deaminase in the reaction framework under consideration. Orthophosphate liberation by myosin was probably minimum because of the absence of calcium ions and the presence of 8.3 mM $MgCl_2$.

If an amount of glucose approximately equivalent to the amount of creatine phosphate originally present was now added, there was a rapid conversion of most of the adenine nucleo-

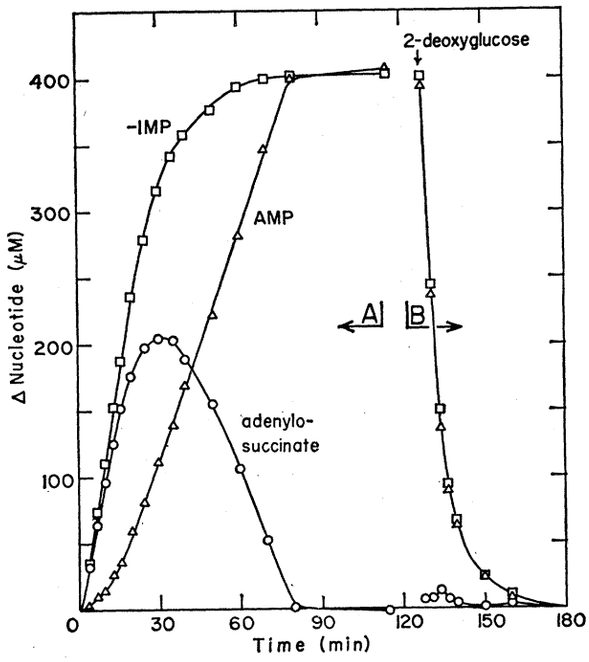

Fig. 3. Concentrations of IMP, AMP (as adenine nucleotide), and adenylosuccinate calculated from the set of spectral scans shown in part in Fig. 2. The first scan ($t = 1$ minute) is used as a base line for part A, and the last scan ($t = 178$ minutes) is used as a base line for part B. Experimental details are given in Fig. 2.

tide to IMP. (Hexokinase was present throughout the experiment.) After a further interval the IMP was converted back to AMP because the glucose 6-phosphate formed was metabolized and yielded the energy necessary for the recovery process. This explanation is confirmed by the observation that addition of 2-deoxyglucose in place of glucose led to the same rapid conversion of adenine nucleotide to IMP, but the conversion of IMP back to AMP now failed to take place. Figure 2B shows several of the eight scans obtained on adding 2-deoxyglucose. The concentrations of intermediates calculated from this series of scans are shown in Fig. 3B. There was little or no difference between the rate of IMP appearance and AMP disappearance. A very small but significant amount of adenylosuccinate was formed and dissipated while the system was running toward IMP. Under these conditions the NTP-regenerating system was exhausted, and GDP accumulated. As has already been mentioned, adenylosuccinate synthetase is strongly inhibited by GDP. Hence the activity of adenylosuccinate synthetase became rapidly inhibited by the accumulation of the reaction product GDP.

The experiments described above show that if an excess of nucleoside triphosphate regenerating substrate is used, the adenine nucleotides become "trapped" in the form of ATP; this state of affairs mimics resting muscle. If, however, a limited amount of a NTP–regenerating substrate is employed in conjunction with a strong ATP drain, then, when the energy-regenerating substrate becomes exhausted, the adenine nucleotides are rapidly converted to IMP. This situation mimics muscle in maximum tetani. In the foregoing experiments with muscle extract, *all* of the adenine nucleotides were converted to IMP; this differs from the normal, physiological tetani in which only small amounts of IMP are formed. Our experiments were designed to demonstrate spectrophotometrically the operation of the individual steps of the purine nucleotide cycle.

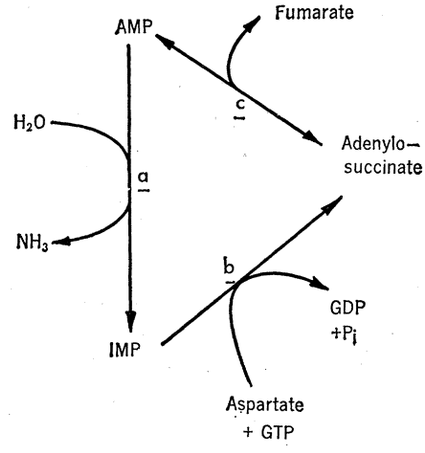

Fig. 4. The purine nucleotide cycle. The enzymes involved in the reactions of the cycle are as follows: (*a*) adenylate deaminase; (*b*) adenylosuccinate synthetase; and (*c*) adenylosuccinase. One turn of the cycle results in the net reaction: aspartate + GTP + HO_2 ⟶ fumarate + GDP + P_i + NH_3.

The experiments shown in Figs. 2 and 3 were continued as follows. When the spectrophotometric scans indicated that the first cycle of the sequence IMP → adenylosuccinate → AMP → IMP was completed, a second portion of creatine phosphate was added to initiate a second cycle. When the spectrophotometric scans indicated that the reamination portion of the second cycle had been completed, 2-deoxyglucose was again added to initiate the deamination portion of the second cycle. The successive additions of creatine phosphate and 2-deoxyglucose were repeated twice more; in each case the times of the additions were judged on the basis of spectrophotometric scans. In this manner the reaction sequence IMP → adenylosuccinate → AMP → IMP was cycled four times in succession. Ammonia analyses of the complete experiment demonstrate clearly that ammonia production depends on the presence of aspartate and IMP (Table 1).

Each complete turn of the purine nucleotide cycle can be expected to result in a maximum production of 1 mole of ammonia per mole of purine nucleotide present. In the experiment recorded in Table 1, four turns of the cycle resulted in the production of 3.2 moles of ammonia per mole of IMP originally present. It follows that the hypoxanthine nucleotide requirement is catalytic and not stoichiometric. Similar experiments showed that the requirement for IMP can also be met by adenylosuccinate and AMP.

The amount of AMP formed from IMP provides another measure of the amount of ammonia which can be expected to be formed during the operation of the purine nucleotide cycle, since this interconversion does not necessarily proceed in 100 percent yield. When the amount of AMP formed during each cycle was determined from the spectrophotometric scans and was compared with the amounts of ammonia produced (Table 1), it was found that 1.1 moles of ammonia was liberated per 1.0 mole of AMP formed from IMP. The slight excess of ammonia formed over AMP formed probably resulted from a small degree of recycling. That this occurred is indicated by the small amounts of adenylosuccinate which were observed to be formed during the deamination portion of the cycle (Fig. 3B).

The experiments presented above constitute strong evidence for the cyclical occurrence of the reaction sequence depicted in Fig. 4. The operation of the

purine nucleotide cycle can explain early experiments with nitrogen-15 ammonia which showed that there is a rapid turnover of the 6-amino group of adenine mononucleotides of striated muscle in vivo, in contrast to a very slow turnover of the ring nitrogens (*14*).

The probable functions of the purine nucleotide cycle fall into several overlapping categories. (i) It is a pathway for the liberation of ammonia from amino acids. Unlike ammonia liberation by way of the glutamate dehydrogenase reaction, ammonia liberation by way of the purine nucleotide cycle is energetically favored. Moreover, muscle either lacks glutamate dehydrogenase or possesses very low amounts of this enzyme. (ii) It is a pathway for the adjustment of the concentrations of citric acid cycle intermediates. Muscle and most other tissues lack pyruvate carboxylase. Malic enzyme has kinetic characteristics that make a net synthesis of malate from pyruvate very unfavorable under most physiological conditions. The aspartate aminotransferase reaction does not lead to a net production of oxaloacetate and α-ketoglutarate. The purine nucleotide cycle provides an alternative pathway for the net supply of four-carbon, dicarboxylic acids to the citric acid cycle. (iii) It is a pathway for regulating the relative concentrations of the adenine nucleotides AMP, ADP, and ATP. Deamination of AMP to IMP leads to a readjustment of the myokinase equilibrium (*15*) (reaction 4)

$$2\ \text{ADP} \longleftrightarrow \text{AMP} + \text{ATP} \qquad (4)$$

toward ATP, with a resulting increase in the concentration ratio of [ATP] to [ADP]. This type of adjustment may be necessary when the resynthesis of ATP from ADP by other pathways is inadequate. (iv) It is a pathway which aids in the control of phosphofructokinase activity. Ammonium ions stimulate phosphofructokinase. In addition, the liberation of ammonia from AMP serves to raise the intracellular *p*H. Inhibition of phosphofructokinase by physiological concentrations of ATP is very pronounced below *p*H 7.1. It is negligible above *p*H 7.3. Full details and documentation for these functions are provided elsewhere (*16*).

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Cyclic Adenosine and Guanosine Monophosphates and Glucagon: Effect on Liver Membrane Potentials

Abstract. *Cyclic adenosine monophosphate, cyclic guanosine monophosphate, glucagon, and isoproterenol each hyperpolarized perfused rat liver cells. The hyperpolarization followed a time course similar to the stimulated increase in potassium efflux and was preceded by the increase in calcium efflux. The hyperpolarization induced by cyclic adenosine monophosphate was blocked by tetracaine. The similarity of the action of the cyclic nucleotides to that of glucagon supports the hypothesis that cyclic adenosine monophosphate is the secondary messenger mediating the action of glucagon.*

Adenosine 3′,5′-monophosphate (cyclic AMP) is thought to be the secondary messenger mediating the action of epinephrine and glucagon on glycogenolysis, gluconeogenesis (*1, 2*), calcium efflux (*3, 4*), and transmembrane potassium flux in the rat liver (*3, 5*). Cyclic AMP also mediates the potassium-dependent beta-adrenergic hyperpolarization in vascular smooth muscle (*6*), and the inhibitory action of catecholamines on the discharge frequency, membrane potential, and membrane resistance of rat cerebellar Purkinje cells (*7, 8*). Guanosine 3′,5′-monophosphate (cyclic GMP) is another naturally occurring nucleotide that has effects similar to those of cyclic AMP on the carbohydrate metabolism of the liver (*9*). The purpose of our study was to determine whether cyclic AMP, cyclic GMP, and the agents that increase endogenous hepatic cyclic AMP concentrations, such as glucagon and the beta-adrenergic agent isoproterenol (*1*), have an effect on the membrane potential of the liver cells. Furthermore, as an effect on the membrane potential became evident, we correlated this with the time course of the ion fluxes induced in the liver by glucagon and cyclic AMP. Finally, since tetracaine, a local anesthetic, blocks the calcium efflux, glycogenolysis, and gluconeogenesis induced by glucagon and cyclic AMP (*4*), we proceeded to establish a similar inhibitory action on the hyperpolarizing effects of cyclic AMP.

Rat livers were perfused in situ with Krebs' bicarbonate solution containing 4 percent albumin and 20 m*M* sodium pyruvate (*4, 10*). Membrane potentials were determined with floating intracellular microelectrodes (*11*), and prior to the addition of drugs were greater (Table 1) than those reported in liver slices, but within the range or slightly below those measured in nonperfused animals (*12*).

Cyclic AMP (1 m*M*), cyclic GMP (0.5 m*M*), or glucagon (0.1 μg/ml) produced significant hyperpolarization (Table 1) after a delay (*13*) of 4 to 8 minutes (Fig. 1). Isoproterenol (0.2 μg/ml) had a similar hyperpolarizing effect that was not abolished by the alpha-blocker, phentolamine (Fig. 1). Isoproterenol also increases the cyclic AMP concentration in the liver (*14*). Adenosine 5′-monophosphate (1 m*M*) in four experiments had no effect on the membrane potential and in another four rats it produced a slight hyper-