(*7*), and all are linked to a marker for tryptophan synthesis (*trp4*) that was carried incidentally in the crosses. The mutations are located about the same distance (averaging 18 centimorgans) from the *trp4* marker on fragment 2 of the *S. cerevisiae* genetic map (Table 1). We also followed the segregation of some of the *asp1* mutations with respect to *trp1*, a centromere-linked gene (*7*). These data indicate that the *asp1* gene is not significantly centromere linked. Therefore, *asp1* is probably not linked to *asp5*, which is located near the centromere of chromosome XII (*4*).

Complementation tests were performed with all pairwise combinations of the 14 mutations to determine whether they are all alleles of the same gene (*8*). From the genetic mapping crosses, we selected segregants that carried the original mutations in combination with the mating type alleles opposite to those of the strains in which the mutations were originally induced. Diploids from 196 pairwise matings were grown and replica plated onto the selective media. All diploids grew well with L-aspartic acid, whereas only certain ones grew with L-asparagine; the pattern of growth was typical of intragenic complementation (*8*) (Fig. 1). The complementation map contains six complementation classes, but only a single functional unit (cistron) is involved, because six of the alleles do not complement any of the others. The fact that complementation does occur indicates that, in *S. cerevisiae*, L-asparaginase is a multimeric enzyme (*8*); the genetic evidence further suggests that only one structural gene for L-asparaginase exists in haploid cells of *S. cerevisiae*.

We also determined some of the biochemical characteristics of yeast L-asparaginase. In crude extracts, the enzyme is stable for at least 2 months when frozen at −25°C and for at least 3 hours at 30°C. Activity decreases logarithmically with time of heating when the extract is held at 50°C and then assayed at 30°C. Enzyme in the crude extract appears to exhibit classical Michaelis-Menten kinetics, the apparent K_m for L-asparagine utilization being about $2.5 \times 10^{-4} M$.

All 14 of the *asp1* mutants have been tested for L-asparaginase activity in vitro and are deficient in the ability to convert L-asparagine into L-aspartate and ammonia. In the presence of unlimited substrate, L-asparaginase activity is usually greater than 5×10^{-2} μmole of ammonia evolved per minute per milligram of protein in extracts from wild-type cells. No activity was detected in extracts from 12 of the mutants, while in extracts of two of them (*asp1-8* and *asp1-10*), the activity was about 10 percent of that measured in the wild-type.

GARY E. JONES
ROBERT K. MORTIMER
Division of Medical Physics, University of California, Berkeley 94720

References and Notes

1. J. Roberts, M. D. Prager, N. Bachynsky, *Cancer Res.* **26**, 2213 (1966); H. A. Campbell, L. T. Mashburn, E. A. Boyse, L. J. Old, *Biochemistry* **6**, 721 (1967).
2. W. F. Geddes and A. Hunter, *J. Biol. Chem.* **77**, 197 (1928); W. Grassmann and O. Mayr, *Hoppe-Seyler's Z. Physiol. Chem.* **214**, 185 (1933).
3. H. de Robichon-Szulmajster, Y. Surdin, R. K. Mortimer, *Genetics* **53**, 609 (1966).
4. R. K. Mortimer and D. C. Hawthorne, *ibid.*, p. 165.
5. Formerly called *thr5* (*4*). The name *asp5* seems more appropriate, because L-aspartate is a branch point in the biosynthetic pathways of several metabolites.
6. G. Lindegren, Y. L. Hwang, Y. Oshima, C. C. Lindegren, *Can. J. Genet. Cytol.* **7**, 491 (1965).
7. D. C. Hawthorne and R. K. Mortimer, *Genetics* **45**, 1085 (1960).
8. J. R. S. Fincham, *Genetic Complementation* (Benjamin, New York, 1966).
9. D. D. Perkins, *Genetics* **34**, 607 (1949).
10. Supported by the U.S. Atomic Energy Commission. One of us (G.E.J.) is sponsored by a predoctoral fellowship from the U.S. Public Health Service.

8 October 1969

Adenohypophysial Transmembrane Potentials: Polarity Reversal by Elevated External Potassium Ion Concentration

Abstract. *Incubation of rat adenohypophyses in potassium ion of sufficient concentration to provoke the release of several of the adenohypophysial trophic hormones produces a reversed, positive transmembrane potential in more than half the cells. This finding is consistent with a process of "stimulus-secretion coupling" in which hypothalamic releasing factors act by selective depolarization of their "target" cells. The positive potentials may be due to a prolonged preferential permeability to calcium ions triggered by an initial depolarization of the cell membrane to a threshold value by increased external potassium ion.*

In experiments on the adrenal medulla and neurohypophysis, Douglas (*1*) found that the sequence of events preceding hormone release closely resembles the sequence of events preceding contraction in skeletal muscle. They proposed a general theory of "stimulus-secretion coupling" whereby hormone release is initiated by a decrease in transmembrane potential followed by an influx of Ca^{2+}. As the theory predicts, the release of several adenohypophysial hormones, thyroid stimulating hormone, luteinizing hormone, and adrenocorticotropic hormone (*2*), is increased by exposing adenohypophyses to elevated potassium ion concentration in vitro. We investigated the transmembrane potential changes accompanying the augmented release of these hormones following exposure to elevated potassium ion concentration.

Adenohypophyses were obtained from young adult male Sprague-Dawley rats (Holtzman) which had been maintained in a constant environment for at least 2 weeks (*3*). The animals were killed by decapitation within 20 seconds after their removal from the cage. Adenohypophyses were quickly removed and transferred to a plexiglass bath through which was circulated either Krebs-Ringer bicarbonate alone (*4*) or a "modified" Krebs-Ringer bicarbonate containing 25 m*M* K^+ with sodium ion concentration appropriately reduced. Solutions were kept at 37°C and gassed with 95 percent O_2 and 5 percent CO_2 to maintain a *p*H of 7.2. Transmembrane potentials were measured with glass micropipettes containing 3*M* KCl. Their resistance was greater than 20 megohms, and the tip potential was insensitive to change in potassium ion concentration. The microelectrode was advanced stepwise along a series of penetration tracks with 10 μm increments that allowed 15 seconds between steps, and the resting potentials were recorded on film. Only those potential changes which were abrupt, which were maintained during the entire interval between advances, and which were greater in magnitude than the base-line shift in potential during the recording of a track were recorded.

Of 70 potentials recorded from 24 tracks in six adenohypophyses during perfusion with Krebs-Ringer bicarbonate (Figs. 1A, 2A, and Table 1), 14 were positive. There was no correlation between depth of penetration and

Table 1. Potentials recorded from adenohypophyses during perfusion with Krebs-Ringer bicarbonate containing either the usual (5 mmole/liter) or an elevated (25 mmole/liter) concentration of K^+. The data are expressed in millivolts plus or minus the 95 percent confidence value.

Type	Number	Average (mv)
*Krebs-Ringer bicarbonate**		
−	56	−12.9 ± 1.8
+	14	4.8 ± 2.0
Total	70	−9.4 ± 2.3
Krebs-Ringer bicarbonate (elevated K^+)†		
−	32	−13.3 ± 2.9
+	49	13.1 ± 2.1‡
Total	81	2.7 ± 3.3‡

* Data from Fig. 1A. † Data from Fig. 1B. ‡ These averages differ significantly (t-test, $P < .05$) from corresponding averages listed above.

magnitude of potentials (Fig. 1A). There was a significant correlation between track length over which a potential was recorded and the magnitude of the potential itself (Fig. 2A). This could imply that the transmembrane potential is proportional to cell size.

Of 81 potentials recorded from 23 tracks in six additional glands during perfusion with Krebs-Ringer bicarbonate with elevated potassium ion concentration (Figs. 1B, 2B, and Table 1), 49 were positive. The mean values were significantly greater than those recorded in Krebs-Ringer bicarbonate alone. In contrast, the negative potentials, although proportionally fewer, were of the same magnitude as those recorded in Krebs-Ringer bicarbonate alone. There was no correlation between depth of penetration and magnitude of these potentials (Fig. 1B), and there was no correlation between track length and magnitude of potential (Fig. 2B).

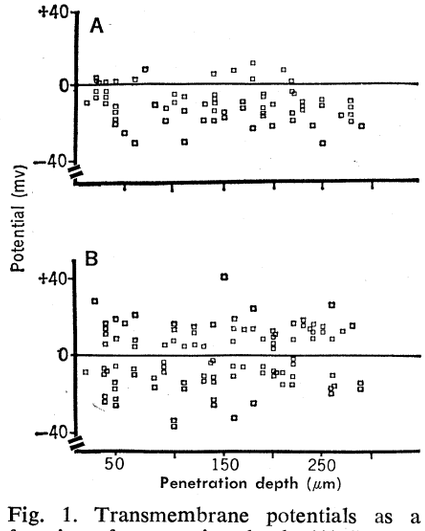

Fig. 1. Transmembrane potentials as a function of penetration depth. (A) Seventy potentials measured during 24 penetrations of the anterior pituitary of six male rats. The perfusing medium was Krebs-Ringer bicarbonate. (B) Eighty-one potentials measured during 23 penetrations of the anterior pituitary of six male rats. The perfusing medium was Krebs-Ringer bicarbonate with elevated concentration of K^+.

In adenohypophysial tissue—unlike nerve or muscle, where elevated potassium ion concentration decreases the negativity of the transmembrane potentials of all the cells (*5*)—only a fraction of cells respond to elevated concentration of potassium ion, and the response of these cells is a reversal of transmembrane potential. The possibility that the positive potentials represent cell injury due to either mechanical damage or anoxia can be excluded since, if this were so, the positive potentials would tend to be confined to the surface of the gland or to cells near the core respectively.

What are the possible ionic mechanisms for the reversal of potentials in high K^+? The transmembrane potential in nerve or muscle can be approximated by the Nernst equilibrium potential of the most permeable ion species (*6*). If K^+ is the most permeable ion, a mean negative potential of 13 mv in the cells of the gland incubated in Krebs-Ringer bicarbonate would indicate an internal K^+ concentration of approximately 10 mmole/liter. Increasing the external concentration of potassium ion to 25 mM would therefore reverse the K^+ gradient and produce positive potentials of about 30 mv. However, we have measured total concentration of K^+ in adenohypophyses (*7*) and have found that, as in many other tissues (*8*), it is approximately 100 mM. This means that the cells of the adenohypophysis must have an intracellular concentration of potassium ion considerably greater than 10 mM. Our findings cannot therefore be explained simply in terms of K^+ distribution.

An alternate hypothesis which can account for the change in polarity of the plasma membrane is related to Ca^{2+} distribution. Elevated concentration of K^+ will increase the permeability of the plasma membrane of the cells of the adenohypophysis to Ca^{2+} (*9*). The concentration of unbound Ca^{2+} is low in most cells; on the order of $10^{-7}M$ in muscle, for example (*10*). If Ca^{2+} were to become the most permeable ion species during the incubation in the high K^+ medium, sizeable positive transmembrane potentials would result. If the muscle value for the internal concentration is used, the equilibrium potential would be about 110 mv, inside positive (*11*).

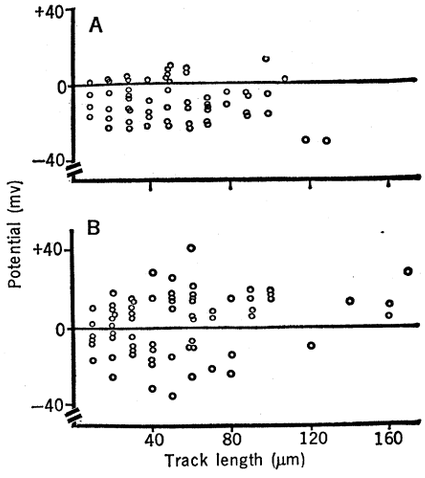

Fig. 2. Transmembrane potentials as a function of track length over which the potential was measured. (A) The data is the same as Fig. 1A. The regression formula for the negative potentials is $Y = -.063\ X - 9.520$. The 95 percent confidence in the slope is ±.057 and in the intercept is ±3.679. The regression formula for the positive potentials is $Y = .070\ X + 1.438$. The 95 percent confidence in the slope is ±.063 and in the intercept is ±3.522. (B) The data is the same as Fig. 1B. The slope of the regression formula was not significantly different from zero for either the negative or positive potentials.

In general our findings have been consistent with a process of "stimulus-secretion coupling." Elevated K^+ concentration, which stimulates release of thyroid stimulating hormone, luteinizing hormone, and adrenocorticotropic hormone (*2*), leads to a reversal of potential in a number of the cells of the adenohypophysis. Since the normal stimulus for release of these hormones is one of a group of hypothalamic releasing factors, we suggest that the releasing factors may initiate release by altering the permeability characteristics of the plasma membrane resulting in depolarization, possibly by preferentially increasing permeability to Ca^{2+}.

J. V. Milligan
Jacob Kraicer
Department of Physiology,
Queen's University,
Kingston, Ontario, Canada

References and Notes

1. W. W. Douglas, *Brit. J. Pharmacol.* **34**, 451 (1968).
2. W. Vale and R. Guilleman, *Experientia* **23**, 855 (1967); M. H. Samli and I. I. Geschwind, *Endocrinology* **82**, 225 (1968); J. Kraicer, J. V. Milligan, J. L. Gosbee, R. G.

Conrad, C. M. Branson, *Science* **164**, 426 (1969).
3. J. Kraicer and S. C. Cheng, *Amer. J. Physiol.* **214**, 158 (1968).
4. W. W. Umbreit, T. H. Burris, J. F. Stauffer, in *Manometric Techniques* (Burgess, Minneapolis, 1964), p. 132.
5. G. Ling and R. W. Gerard, *Nature* **165**, 113 (1950).
6. A. L. Hodgkin, *Proc. Roy. Soc. Ser. B Biol. Sci.* **148**, 1 (1958).
7. Adenohypophyses were removed, weighed, and dissolved in HNO_3; the $[K^+]$ was determined with a flame photometer after appropriate dilution.
8. W. S. Wilde in *Mineral Metabolism* (Academic Press, New York, 1962), vol. 2A.
9. J. V. Milligan and J. Kraicer, unpublished results.
10. A. Weber, R. Herz, I. Reiss, *J. Gen. Physiol.* **46**, 679 (1963).
11. Since Ca^{2+} is divalent, the Nernst equation has the form $E = 26 \times \log_{10} (C_o/C_i)$.
12. We thank C. Ripley and A. Morris for technical aid. J. L. Gosbee assisted with the removal of adenohypophyses. Supported by McLaughlin Research Fund (Queen's) and M.R.C. (Canada).

4 September 1969

Reactivity in vivo of the Carcinogen N-Hydroxy-2-acetylaminofluorene: Increase by Sulfate Ion

Abstract. *Injections of sulfate ion in rats given the carcinogen* N-*hydroxy-2-acetylaminofluorene increased (i) the formation of 1- and 3-(methion-*S*-yl)-2-acetylaminofluorene bound to protein in the liver, (ii) the formation of fluorenyl derivatives bound to total protein, ribosomal RNA, and DNA in the liver, and (iii) the toxicity of the carcinogen. These data provide evidence that the highly reactive ester 2-acetylaminofluorene-*N*-sulfate, previously suggested as an ultimate reactive and carcinogenic metabolite of* N-*hydroxy-2-acetylaminofluorene, is formed in the rat liver in vivo.*

The very low nonenzymatic reactivity of *N*-hydroxy-2-acetylaminofluorene (*N*-hydroxy-AAF) with tissue nucleophiles in vitro (*1*) suggests that metabolic activation is required to convert this carcinogen into an ultimate reactive and carcinogenic form. The possible nature of this reactive form was suggested by findings that synthetic electrophilic esters of *N*-hydroxy-AAF react readily at neutrality with nucleic acids, proteins, and certain of their nucleophilic components (*1–4*). Furthermore, the products formed by reactions of guanosine and methionine with synthetic esters of *N*-hydroxy-AAF, such as *N*-acetoxy-AAF or AAF-*N*-sulfate (Fig. 1), are identical to products obtained on degradation of liver RNA and protein from rats given AAF or its *N*-hydroxy metabolite (*1, 5, 6*). The ester-like O-glucuronide of *N*-hydroxy-AAF, a major urinary and biliary metabolite (*7*), also reacts in similar ways with the same nucleophiles in vitro, but at a much slower rate (*8*).

The supernatant fraction of homogenates of rat liver contains sulfotransferase activity capable of transferring the sulfonate group from 3′-phosphoadenosine-5′-phosphosulfate to *N*-hydroxy-AAF to form AAF-*N*-sulfate (*6, 9, 10*). Further, on the basis of close correlations between sulfotransferase activity for *N*-hydroxy-AAF in the liver, the amount of hepatic protein-bound 1- and 3-(methion-*S*-yl)-AAF formed from *N*-hydroxy-AAF in vivo, and susceptibility to hepatic carcinogenesis, AAF-*N*-sulfate appears to be an ultimate reactive carcinogenic metabolite of *N*-hydroxy-AAF (*6*). Our results provide evidence for the formation and reactivity of this ester in rat liver in vivo.

The instability of AAF-*N*-sulfate (half-life in water of less than 1 minute) (*4*) precludes its isolation from liver, and the loss of sulfate ion on reaction with nucleophiles makes it impossible to infer its presence from the structures of reaction products. However, the enzymatic formation of AAF-*N*-sulfate in vitro is dependent on 3′-phosphoadenosine-5′-phosphosulfate (*6, 9, 10*), and the data of Büch *et al.* (*11*) indicate that the amount of sulfate ion in vivo is a limiting factor in the formation of *p*-acetylaminophenyl sulfate from *p*-hydroxyacetanilide in the rat. Thus our attention was directed to the effect of sulfate ion on the reactivity of *N*-hydroxy-AAF in the liver of the rat, especially in rats given prior treatment with *p*-hydroxyacetanilide to deplete the pool of sulfate ion in vivo.

Male rats (250 to 300 g, CD-randombred, Charles River Breeding Laboratory, Wilmington, Mass.) were injected according to the schedules in Table 1 and Fig. 2. The amount of 1- and 3-(methion-*S*-yl)-AAF bound to protein in the liver was estimated from the

Table 1. Stimulation by sulfate ions of binding in vivo of fluorenyl derivatives of *N*-hydroxy-AAF to protein-bound methionine or to the total protein, ribosomal RNA, and DNA in rat liver. In experiment 1, *p*-hydroxyacetanilide (19 mg/100 g of body weight; in 1*M* NaCl or 0.5*M* Na_2SO_4) was injected at 0 and 3 hours; *N*-hydroxy-AAF was injected at 4 hours. In experiment 2, *p*-hydroxyacetanilide (19 mg/100 g of body weight; in 2*M* NaCl, 1*M* Na_2SO_4, 1*M* sodium phosphate, or 2*M* sodium acetate, all at *p*H 7) was injected at 0 hour; *N*-hydroxy-AAF was injected at 4 hours. In experiment 3, water, 1.5*M* NaCl plus 0.25*M* $MgCl_2$, or 0.75*M* Na_2SO_4 plus 0.25*M* $MgSO_4$ were injected at 0 hour; *N*-hydroxy-AAF was injected at 3 hours. In experiment 4, the injections were as in experiment 3, except *N*-hydroxy-AAF was injected at 2 hours. In experiment 5, the injections were as in experiment 2, except C^{14}-labeled *N*-hydroxy-AAF was injected at 4 hours. All injections were intraperitoneal (0.5 ml/100 g of body weight). The *N*-hydroxy-AAF was suspended in 1.75 percent gum acacia, which in experiments 1, 3, and 4 contained the same concentrations of salts as the solutions injected at 0 time. The differences between the amounts of bound fluorenyl derivatives for the livers of rats injected with sulfate or chloride ions had *P* values of < 0.05 for DNA and ribosomal RNA and of < 0.01 for protein (Student's *t*-test). The results are expressed as the mean ± 1 standard deviation; numbers of rats are in parentheses.

Expt.	*N*-hydroxy-AAF (mg/100 g)	Time rats killed after *N*-hydroxy-AAF injection (hr)	Amount of fluorenyl derivatives with injection of		
			Sulfate	Chloride	No anion
			Bound to methionine in protein†		
1*	5	16	29 ± 6 (6)	8 ± 4 (6)	
2*	5	16	38 ± 8 (5)	14 ± 3 (6)‡	
3	12	2	78 ± 19 (13)	55 ± 13 (7)	51 ± 23 (6)
4	20	2	98 ± 14 (4)	63 ± 19 (4)	68 ± 12 (4)
			Bound to protein§		
5*	5	16	1470 ± 220 (5)	780 ± 140 (5)	
			Bound to ribosomal RNA§		
5*	5	16	325 ± 90 (5)	155 ± 70 (5)	
			Bound to DNA§		
5*	5	16	280 ± 95 (5)	200 ± 70 (5)	

* Given prior treatment with *p*-hydroxyacetanilide. † Expressed as micrograms of *o*-methylmercapto-AAF liberated per 5 g of liver. ‡ When phosphate or acetate ions were injected in place of chloride ions, the values were 12 ± 1 (3) and 11 ± 3 (3), respectively. § Expressed in picomoles per milligram.