3 percent; butyl acetate, 1 percent; amyl acetate, 2 percent; 4-heptanone, 0 percent; geraniol, 0 percent; butanol, 8 percent; benzaldehyde, 3 percent; methyl benzoate, 4 percent; and furfurol, 5 percent (*8*). These variations demonstrate that, within any one animal, odorant retention times are highly consistent in almost all cases.

Figure 1 demonstrates that, for most odorants, concentration does not appear to be a major determinant of relative retention time across the mucosa. However, there are some odorants that do show at least a suggestion of a concentration effect. This may explain why in an earlier electrophysiological study the LB/MB ratios produced by two of these odorants, butyl acetate and heptaldehyde, were observed to increase slightly with increasing concentration (*4*).

Of prime consideration in Fig. 1 is the range of different relative retention times produced by different odorants. Using all ten animals this range shows an approximate 220-fold increase from the shortest to the longest and the remaining relative retention times appear rather well distributed within these limits. This range represents an increase from a mean of 1.2 seconds to a mean of 274 seconds. Thus in spite of its small size the molecules of different odorants do appear to migrate at significantly different rates across the in vivo olfactory mucosa of frog.

Since we now have direct measurements of the facility with which molecules of different odorants migrate across the mucosa, we might ask whether the LB/MB ratios which originally led Mozell to support a chromatographic model of olfactory discrimination are in fact related to this facility. We plotted the mean LB/MB ratios (*4*) against the mean relative retention times across the mucosa determined for all ten animals (Fig. 2). There is an inverse relationship, which is given statistical emphasis by a highly significant ($P < .001$) rank order correlation coefficient ($r_s = -.81$). Thus the earlier supposition that the LB/MB ratios reflect the differential migration of molecules across the mucosa is supported by these direct measurements of the behavior of the molecules themselves. As additional evidence for this conclusion, the butanol LB/MB ratio that appeared to deviate from the general trend when plotted against its retention time on a Carbowax column (*4*) falls more nearly into its predicted position when plotted against its relative retention time along the mucosa.

From the correlation discussed above we might expect a direct relationship between the retention times previously measured across Carbowax (*4*) and those measured across the mucosa. The inset of Fig. 2 shows the strength of this relationship. Thus, some of the same properties which underlie and characterize the chromatographic behavior of a Carbowax column may also underlie and characterize the observed chromatographic behavior of the olfactory mucosa (*3, 4*).

We must emphasize that this analogy between olfaction and chromatography is made only in regard to the basic principle involved (the analysis of chemicals by the propensity of their molecules to migrate at different rates along a medium) and not to any particular set of operations currently used in any laboratory application of that principle. Presumably the nose would develop operational details, in order to take advantage of this principle, which are compatible with its own peculiarities and requirements and which would not necessarily mimic any other adaptation of the same chromatographic principle (*3, 4, 9*). For instance, as a result of respiration the carrier gas flows through the nose in two directions and is pulsatile rather than, as in a standard gas chromatograph, unidirectional and constant. Consequently, rather than using a measure based upon the time needed for molecules to travel the given distance to the one detector at the end of the column, the nose could measure, with its many detectors spread along its entire column, the distance the molecules travel and the relative number of molecules traveling that distance.

We have demonstrated that, in spite of the small size of the mucosa, the molecules of different odorants do indeed differ significantly in their ability to migrate along it. This chromatographic differentiation may be one of the mechanisms underlying olfactory discrimination. However, even if this is not so, its demonstrated existence still has major implications. For instance, the molecules of low vapor pressure odorants, which generally have long retention times, will, in a given sniff, be piled up near the entrance to the olfactory sac. They may not reach mucosal regions farther along the nasal flow path. Consequently, a large fraction of the olfactory receptors may not contact the incoming molecules of such chemicals. On the other hand, for those receptors near the entrance, which do make contact, the concentration of these molecules will be greatly increased. Such phenomena must be considered in our further attempts to understand olfactory processes.

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16 February 1973; revised 21 May 1973

Recombinant Inbred Lines: Value in the Genetic Analysis of Biochemical Variants

Abstract. *Analysis of inducibility by androgens and electrophoretic mobility of kidney glucuronidase in progenitor and derived recombinant inbred mouse lines suggests that a single major regulatory gene at or near the glucuronidase structural gene on chromosome 5 determines the rate of enzyme accumulation.*

The utility of recombinant inbred (RI) lines of mice for the analysis of histocompatibility gene systems (*1*) and plasma corticosterone (*2*) has been demonstrated. We now report their special utility in the genetic analysis of a complex biochemical phenotype, the regulation of enzyme induction by a hormone.

The RI lines are produced by inbreeding the F_2 generation of a cross between two unlike progenitor inbred

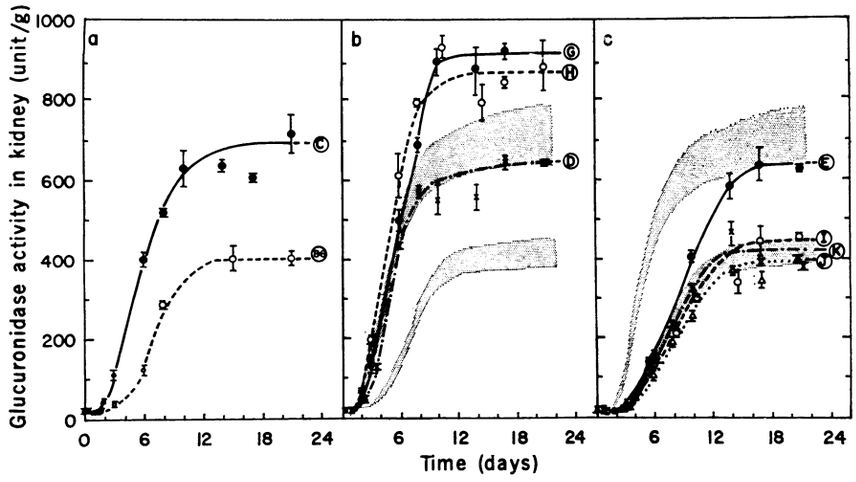

Fig. 1. Induction of kidney β-glucuronidase in progenitor and recombinant inbred mouse lines by dihydrotestosterone. Female mice, 3 to 4 months of age, were injected subcutaneously on day zero with 10 mg of 5α-dihydrotestosterone (Sigma) prepared as a homogenized suspension (50 mg/ml in olive oil) and again on day 1, and every third day thereafter with 5 mg. Every sixth day, two animals of each line were killed, and their whole kidneys were homogenized in 19 volumes of 0.25*M* sucrose containing 0.02*M* imidazole-HCl at *p*H 7.4 with a Polytron homogenizer (Kinematica GMBH Lucerne) for 1 minute. The specific activity of kidney β-glucuronidase (*5*) is plotted as the mean of the assay value of two animals (horizontal bars). One unit of enzyme is the amount that produces 1 μmole of *p*-nitrophenol per hour at *p*H 4.6 with 1.0 m*M* *p*-nitrophenyl β-D-glucuronide as the substrate. (a) Progenitor lines C and B6. (b) High inducible RI lines D, G, and H. (c) Low inducible RI lines E, I, J, and K. The shaded areas in (b) and (c) represent the induction values expected for progenitor lines C and B6, as calculated from the maximum variation of values shown in (a).

strains. Inbreeding of the independent RI lines produced from such a cross results in segregation and recombination from the F_1 generation until the RI lines approach full homozygosity. One can estimate the number of genes responsible for a biochemical trait if the expression of that trait differs in the two progenitor lines. If all RI lines have phenotypes that are identical to either one or the other progenitor, it is likely that a single gene is responsible. If new phenotypes are present, they represent the occurrence of new genetic combinations; from the number and nature of the new phenotypes one may deduce the minimum number of genes involved. As a test of genetic linkage, the distribution among the RI lines of alleles at a new locus can be compared with the distribution of alleles of other known loci. The more closely two genes are linked, the more similar their distribution patterns are likely to be.

The RI lines provide a unique advantage in the analysis of complex biochemical phenotypes in that they permit the detailed genetic study of the complete time course of a process such as enzyme induction. Once an RI line has been established, an indefinite supply of animals of identical genotype are available. In conventional genetic crosses, each offspring is unique and can be analyzed destructively only once.

We have used RI lines to examine genetic differences in androgen inducibility of kidney β-glucuronidase (E.C. 3.2.1.31). Glucuronidase induction is confined to the proximal convoluted tubules. When stimulated by androgens these tubules hypertrophy but do not divide or synthesize appreciable DNA (*3*). The induction of β-glucuronidase activity is due to de novo enzyme synthesis as evidenced by the incorporation of radioactive amino acids into antibody purified enzyme after the cells are exposed to androgens (*4*). A survey of β-glucuronidase induction in inbred mouse strains has shown that the strains can be divided into two principal classes; the enzymes of one group, the high inducing strains, induce much faster and obtain greater activity levels than those of the other group, the low inducing strains. One of the high inducing strains is BALB/c and one of the low inducing strains is C57BL/6. At present, seven RI lines derived from a cross of BALB/cBy (C) with C57BL/6By (B6) are available, and we have used them to determine which characteristics of the β-glucuronidase induction curve are inherited together and to estimate the number of genes involved. The seven RI lines (D, E, G, H, I, J, and K) have undergone more than 40 generations of full sibmating (*1*).

The β-glucuronidase activities in the progenitor lines C and B6 are identical before the administration of androgens (Fig. 1a); glucuronidase specific activity values as assayed in whole kidney homogenates were 24.7 ± 0.8 (S.D.) unit/g in four C controls and 25.0 ± 1.1 unit/g in five B6 controls. Between days 2 and 10 after androgen administration there is a rapid rise in enzyme activity, but the rise is much faster in C than in B6. Also, the lag period before enzyme activity has increased so that it is significantly greater than activity in uninduced cells is only 30 to 37 hours in C but is 43 to 48 hours in B6. On day 10 after induction, β-glucuronidase specific activity reaches a level of 600 to 700 unit/g in C and 400 unit/g in B6 and the enzyme activity remains constant thereafter.

The kidney glucuronidase activities in uninduced females of all seven RI lines were not significantly different from the levels in the uninduced progenitor lines. When RI lines D, H, and G were induced for kidney glucuronidase (Fig. 1b), they showed a lag period similar to that of the C progenitor and accumulated enzyme from days 2 to 8 at the same rate as the C progenitor. However the final enzyme activities of two of these RI lines, G and H, were significantly higher than that of either progenitor. The existence of these "nonprogenitor" RI lines must result from a new combination of progenitor genes. It is, therefore, very likely that at least two genes are important in determining the final activity of the enzyme.

When glucuronidase was induced in the remaining four RI lines, E, I, J, and K (Fig. 1c), there was a lag period and rise very similar to that of the B6 progenitor (Fig. 1a). The final enzyme activity, however, of one line, E, was 50 percent greater than that of B6 and only slightly lower than that of C. The existence of a new phenotype with a rate of induction like the B6 progenitor and a final enzyme activity similar to the C progenitor is further evidence that more than one gene determines

the shape of the enzyme induction curve.

The minimum number of genes responsible for determining the shape of the entire induction curve can be estimated by comparing the curves for the progenitors and the RI lines (Fig. 1). It is likely that one gene is responsible for determining the lag and rise portions of the curve since all RI lines resemble either one or the other parent early in the process of induction. To account for the four classes of RI lines distinguishable at the plateau period of induction from days 10 to 21, one must postulate at least two genes, each with different progenitor alleles. Presumably these genes have recombined in lines G and H and in line E. If the gene controlling lag and rise values is one of the two genes affecting the plateau region, then a two gene difference in the progenitor lines could explain the complete time course of induction for all RI lines.

Several laboratories have discovered that there are two classes of β-glucuronidase distributed among inbred mouse lines. The two classes are differentiated by electrophoretic mobility of the enzyme at neutral *p*H (*6*, *7*). The progenitor lines C and B6 are homozygous for the faster and slower moving electrophoretic alleles, respectively, and so it was possible to test for genetic linkage of enzyme inducibility and electrophoretic mobility among the seven RI lines (Fig. 2). We found that RI lines with a slow rate of enzyme increase (E, I, J, and K) are homozygous for the B6 electrophoretic form of glucuronidase, whereas the three RI lines with a rapid rate of induction (D, G, and H) are homozygous for the C electrophoretic allele. If the genes for inducibility and electrophoretic mobility were not on the same chromosome, the probability that they would assort identically in all seven RI lines is $1/2^7$ or less than 1 percent.

These experiments suggest that in the mouse a single gene controls both the lag period before the androgen-induced increase in kidney glucuronidase activity and the rate of increase of the enzyme activity. Furthermore, this gene is associated with the electrophoretic mobility of the glucuronidase. The genetic locus that specifies the enzyme electrophoretic alleles is almost certainly the structural gene for glucuronidase. Alleles at this locus segregate codominantly, and the locus is closely linked to the locus on chromosome 5 of the glucuronidase structural gene that determines enzyme thermostability (*4*, *12*). Thus it appears that inducibility is controlled by a site at or near the locus of the enzyme structural gene. The fact that strains with different inducibilities still have the same enzyme levels in kidney and in other tissues (*4*) before induction suggests that there may be a regulatory locus separate from the enzyme structural gene.

The results of a conventional genetic analysis have confirmed our results with RI lines (*4*). That is, it is likely that a single gene closely linked to the glucuronidase structural gene controls the slope of the enzyme induction curve. Moreover, rates of protein synthesis suggest that this gene regulates enzyme activities by altering the rate of synthesis of glucuronidase (*4*). The conventional genetic study, in fact, was aided by the prior analysis of the RI lines, which suggested that the inducibility difference between high and low lines was genetically least complex along the rise portion of the curve.

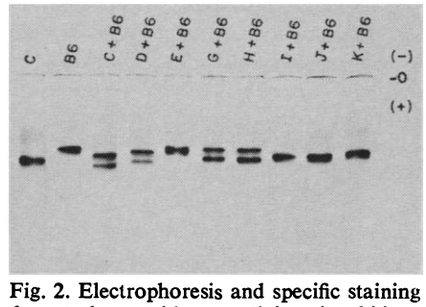

Fig. 2. Electrophoresis and specific staining for β-glucuronidase activity in kidney extracts of progenitor and recombinant inbred lines. The kidney extracts were made from mice induced for 14 days by 5α-dihydrotestosterone. Triton X-100 was added to make a solution of 95 percent kidney extract and 5 percent detergent. For electrophoresis, extracts from RI lines E, I, J, and K were mixed with equal volumes of B6 extract containing glucuronidase of slow mobility, while samples from parental lines C and RI lines D, G, and H were mixed with twice their volume of B6 extract. We applied 10 μl to each gel. The electrophoresis system (*8*) was modified so that the buffer reservoirs contained 5 m*M* TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] plus 5 m*M* HCl titrated to *p*H 7.0 with imidazole. The 6 percent polyacrylamide gels contained 12.5 m*M* TES titrated to *p*H 7.2 with imidazole. Electrophoresis was toward the anode at 50 volt/cm for 90 minutes. Gels were specifically stained for glucuronidase activity by a simultaneous dye coupling method (*9*), with naphthol-AS-BI-β-D-glucuronide as substrate. The method was modified by decreasing the *p*-rosaniline concentration to 0.3 m*M* to decrease background staining. The most rapidly migrating band in all extracts is lysosomal glucuronidase; the fainter bands visible in some gels closer to the cathodal end of the gel are of microsomal origin. Microsomal glucuronidase is encoded by the same structural gene as lysosomal glucuronidase (*10*) and is induced in parallel with lysosomal glucuronidase (*11*).

The low inducibility allele present in the B6 progenitor appears similar to the operator noninducible mutation that results in mouse kidney glucuronidase with lowered inducibility and slow electrophoretic mobility (*13*). There is an important difference, however, in that the F_1 progeny of the low and high progenitors studied here had additive glucuronidase values throughout induction (*4*) while the operator noninducible variant has been reported to be recessive (*13*).

The RI lines are extremely useful for the genetic analysis of complex biochemical processes whose study requires replicate analyses at many different times. In this regard, they are admirably suited for the analysis of patterns in development and differentiation as well as the kinetics of hormone induction since the same recombinant population can be measured at different stages in development or at different times after receiving a stimulus. As the number of gene markers typed in the RI lines and the number of RI lines themselves increase (*14*), it will be possible to determine not only gene segregation but also gene linkages simply by typing each RI line for the new marker and comparing the strain distribution pattern with those already recorded for other genes. These considerations along with the potential use of RI lines in other types of studies, including mutation analysis (*1*, *14*), emphasize their utility as a potent analytical tool in mammalian biochemical genetics.

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15. We thank M. Batt for technical assistance. Supported in part by NIH research grants GM-15574 and CA-12663 (to D.W.B.) and GM-18484-02 and GM-19521-01 (to R.T.S.).

14 June 1973; revised 30 July 1973

Biosynthesis of a Vasotocin-Like Peptide in Cell Cultures from Pineal Glands of Human Fetuses

Abstract. *Cultured cells from pineal glands of human fetuses release into their media a substance that has antidiuretic and hydroosmotic activities. The ratio of these activities as well as their susceptibility to tryptic digestion, specific oxidative inactivation by tyrosinase, and reductive inactivation by sodium thioglycollate, indicates the presence of a basic peptide, presumably identical with arginine vasotocin. The total amount of this peptide released into the culture media during 38 days of incubation is about ten times greater than the amount contained in nonincubated pineal glands from fetuses of the same age, strongly suggesting that fetal ependymal cells from the pineal gland can synthesize in vitro a peptide similar to arginine vasotocin.*

Katsoyannis and du Vigneaud's first synthesis (*1*) of arginine vasotocin (AVT) in 1958 (an example of the synthesis of a hormone before it was identified as a natural product) led to the discovery that this is a widespread octapeptide hormone, present in all species so far investigated, from the most primitive living vertebrates (*2*) to man (*3*). In mammals, we first identified AVT in the pineal gland (*4, 5*), and this conclusion has been directly confirmed by the chemical analysis of pineal AVT (*6*). The extraordinary evolutionary stability of AVT suggests that this ancestral molecule may be elaborated by some primitive structure common to all vertebrates. Pavel (*7*) has suggested that pineal AVT may be elaborated by secretory ependymal cells, the most primitive secretory cells of the brain (*8*). If this postulate is true, then ependymal secretory cells from the pineal gland, when cultured in vitro, should have the ability to synthesize AVT. Because, in the fetal pineal gland of mammals (*9*) including man (*10*), the only secretory activity so far detected is located in ependymal cells, we therefore studied human fetuses. Male fetuses, aged 95 to 115 days after ovulation, were obtained aseptically within 15 to 30 minutes of therapeutic abortion. The pineal glands of six fetuses were extracted in 0.25 percent acetic acid as described (*7*) and served as controls for comparison prior to incubation. The pineal glands from four other fetuses were used for cell cultures. Brain tissue (from the region of the frontal lobe) of the same fetuses was used as control tissue in cell cultures. The pineals and neural tissue used for cell culture were minced to provide a crude dissociated cell suspension. The cells and cell clumps corresponding to each gland were suspended in 1 ml of Hanks medium supplemented with 10 percent calf serum and 2.5 percent N16 medium. The culture tubes contained 1 ml of medium and were maintained in a nearly horizontal position at 37°C and *p*H 7, without rocking, for 6 to 8 days. Cell attachment to the bottom of the tubes was complete within 24 hours. On day 8, when the cell cultures were established, the media were first changed, and the tubes were inserted in a rocker incubator system. The culture media were changed every 5 days thereafter. The assay methods used have been described (*4*), and include rat antidiuretic, rat uterus, and frog bladder (*Rana temporaria*) assays. The U.S.P. Posterior Pituitary Reference Standard was used as standard in antidiuretic assays. Synthetic oxytocin (Syntocinon, Sandoz) restandardized against the U.S.P. Standard, was used as standard in hydroosmotic assays. Synthetic AVT (provided by M. Bodanszky, Case Western Reserve University, Cleveland) was used for the calculation of the hydroosmotic rat antidiuretic ratio. Highly purified crystalline trypsin (twice crystallized and chymotrypsin free, salt free, lyophilized; Serva laboratories), tyrosinase (salt free, lyophilized; Worthington Biochemicals), and sodium thioglycollate (British Drug House) were used for tryptic digestion, specific oxidative inactivation, and reductive inactivation, respectively. Every 5 days, the culture media from the pineal cultures from the four fetuses were pooled, adjusted to 4 ml, and assayed for antidiuretic or hydroosmotic activities. No rat uterus activity could be detected in the culture media (that is, less than 25 μunit/ml). Hydroosmotic and antidiuretic activities of the culture media are shown in Table 1. The culture media from day 18 were pooled, and hydroosmotic activity was determined after it was incubated with trypsin and tyrosinase. One milliliter of culture media was incubated with 20 μg of trypsin at *p*H 8 and 38°C for 3 hours. The other half was incubated at the same temperature and *p*H without trypsin. For specific oxidative inactivation, 1 ml of culture media was incubated for 1 hour at *p*H 7.5 and 37°C with tyrosinase (25 μg/ml). The other half was incubated at the same temperature and *p*H without tyrosinase. The hydroosmotic activity was nearly completely destroyed after trypsin and tyrosinase incubation; that is, when the control water flux was 3 μl per 20 minutes, the fluid movement in response to untreated medium was 34 μl per 20 minutes, whereas the response to the medium treated with trypsin or tyrosinase was 6 μl and 5 μl per 20 minutes, respectively. Both the controls and

Table 1. Hydroosmotic and antidiuretic activities of cell culture media from pineal glands of human fetuses expressed in U.S.P. microunits per milliliter ± the standard error.

Day	Activity	
	Hydroosmotic	Antidiuretic
8	3750 ± 634	
13		19 ± 3
23	3100 ± 428	
28		18*
33		20*
38	3480 ± 458	
Mean	3440	19

* The antidiuretic value of day 28 and day 33 represents the equivalent of a single assay used as control in inactivation studies.