TABLE I

REACTION OF OXIDES WITH LIQUID N_2O_4

Oxide	Reaction time, hr.	Oxide reacted, %	NO_2 in product, %	Metal(II) cation in product, %
PbO	7	84	Nil[a]	
Pb_3O_4	9	86[b]	Nil	
PbO_2	7	97	Nil	
Ag_2O	11	100	2.06	
ZnO	6.5	100	37.84[c]	19.14[c]
CdO	7	80	1.61	
MgO	6.5	90	26.84[c]	11.96[c]
CaO	6	50	0.81	
$Ca(OH)_2$[d]	350 days[e]	100	Nil	
SrO	14.5	0	0.22	
BaO_2	8	25	.13	
BaO[f]	14.5	99	.24	
CuO	14.5	85	31.94[c]	22.95[c]
Cu_2O	7.5	100	32.03	22.43
HgO	400 days[e]	100	19.53	47.95
Hg_2O	16 days[e]	100	16.87	

[a] Nil = 0.1% or less by weight of NO_2. [b] Residual oxide Pb_3O_4 by X-ray diffraction pattern. [c] Corrected for unreacted oxide in product. [d] $Ca(NO_3)_2 \cdot H_2O$ isolated: Found: H_2O, 9.52. Theory: H_2O, 9.88. [e] Time (in days), indicates the time the oxide remained in contact with N_2O_4 prior to analysis rather than the time for actual reaction. Observable reaction occurred in these cases in from 4 to 48 hours. [f] 71% $Ba(OH)_2$.

Results and Discussion

The data in Table I are representative of several experiments for each of the listed oxides, prepared at low temperatures. Confirming previous observations,[4,6] the oxides prepared at higher temperatures were much less reactive.

The catalytic effect of water on oxide–liquid N_2O_4 reactions is illustrated by the data for $Ca(OH)_2$ and BaO (71% $Ba(OH)_2$).

The products were either the corresponding anhydrous nitrates or the NO_2 addition compounds of the following analytical composition: $Mg(NO_3)_2 \cdot NO_2$, $Zn(NO_3)_2 \cdot 2.6$ to 3.3 NO_2, $Cu(NO_3)_2 \cdot 2NO_2$ and $Hg(NO_3)_2 \cdot 2NO_2$. These addition compounds were decomposed to the anhydrous nitrates at 10^{-5} mm. and at temperatures ranging from 90 to 140°, depending upon the thermal stability of the particular compound. The preparation of $Mg(NO_3)_2$ was not particularly successful due, we believe, to a concurrent decomposition of the $Mg(NO_3)_2$.

Zinc oxide heated in the presence of liquid dinitrogen tetroxide gave as a product a light brown, viscous oil, which solidified completely to a light yellow, waxy solid in approximately eight hours. The same solid product was obtained with zinc oxide and liquid dinitrogen tetroxide at room temperature. We believe $Zn(NO_3)_2 \cdot 2.6$ to 3.3 NO_2 represents a mixture of $Zn(NO_3)_2 \cdot 2N_2O_4$ + $Zn(NO_3)_2$ isolated at 30° whereas Addison[5] obtained $Zn(NO_3)_2 \cdot 2N_2O_4$ at 15°. In this series of addition compounds, $Zn(NO_3)_2 \cdot 2N_2O_4$ is intermediate in thermal stability between $Cu(NO_3)_2 \cdot 2NO_2$ and $Hg(NO_3)_2 \cdot 2NO_2$.

The copper(I) oxide exhibited a rather unusual behavior toward liquid dinitrogen tetroxide. At room temperature there was no observable reaction other than a slight darkening of the oxide. At 87°, however, the solid product increased in bulk volume some tenfold over that of the original oxide, producing a jade green micro-crystalline solid. In all of these experiments a considerable excess of dinitrogen tetroxide liquid was present after reaction, but in the case of the copper(I) reaction, only about 0.5 ml. of the 20 to 30 mole excess was observed as free liquid. Apparently the reaction product has the ability to absorb a large quantity of the excess dinitrogen tetroxide present; however, no reduction in the bulk volume of the solid product was observed when the excess liquid dinitrogen tetroxide was pumped off. It is interesting to note that the reaction product of copper(II) oxide did not exhibit this property.

Mercury(I) and (II) oxides and anhydrous mercury(II) nitrate reacted with the liquid dinitrogen tetroxide to produce brown viscous oils which were immiscible in the solvent. The oily product could be frozen with liquid nitrogen to a yellow glass-like solid having a conchoidal fracture. The softening point of this material was slightly below the freezing point of liquid dinitrogen tetroxide. $Hg(NO_3)_2 \cdot 2NO_2$ remained as an oil at 25 to 30° in a nitrogen dioxide gas atmosphere of 750 mm., but lost NO_2 very rapidly below this pressure at 30°, yielding a pale yellow solid having a composition approximating $Hg(NO_3)_2 \cdot 0.7NO_2$. This latter product when heated gave mercury(II) nitrate. The reaction of anhydrous mercury(II) nitrate with liquid dinitrogen tetroxide was carried out for the purpose of confirming the composition of the oily product obtained for the oxides. $Hg(NO_3)_2 \cdot 2NO_2$ is the most unstable of these nitrogen dioxide-containing compounds investigated to date. $Hg(NO_3)_2 \cdot 2NO_2$ may be the same compound reported by Boh[4] as his unidentified product obtained from the reaction of mercury(II) oxide and dinitrogen trioxide.

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Divergent Electrophoretic Properties of Dissolved and Adsorbed Trypsin

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There is good experimental evidence that the electrophoretic mobilities of inert particles suspended in a protein solution (microscope method) are often not greatly different from the mobility of the dissolved protein, as determined by the moving boundary method under comparable conditions of *p*H, ionic strength, etc.[1] However, large differences have been noted with several proteolytic

(1) L. S. Moyer, *J. Biol. Chem.*, **122**, 641 (1938); H. A. Abramson, L. S. Moyer and M. H. Gorin, "Electrophoresis of Proteins," Reinhold Publ. Corp., New York, N. Y., 1942.

enzymes. In the case of pepsin, Herriott, Desreux and Northrop[2] found this result "to be due to the effect of decomposition products on the measurement" made by the microscope method.

The experiments to be described here are concerned with the cause of such differences in the case of trypsin. Northrop and Kunitz[3] found the isoelectric point of collodion particles suspended in a solution of trypsin to be "approximately 7 in 0.02 *M* phosphate buffer and approximately 6 in 0.02 *M* acetate buffer." Bier and Nord[4] gave the isoelectric point (pI) as 10.8 for solutions of this enzyme in 0.04 *M* glycine buffer with added $CaCl_2$, 0.03 *M*. They also reported the presence in such solutions of an enzymatically inactive component of low mobility at *p*H 7.5, which consists, in their view, of "inhomogeneous low molecular weight trypsin-split products." They suggest that the results of Northrop and Kunitz "might well be due to interference of trypsin-split products." It seemed worthwhile, as part of a more extended investigation of the electrophoresis of dissolved and adsorbed proteins, to see whether the apparent change in the pI of trypsin when adsorbed could be attributed to selective adsorption of Bier and Nord's inactive component.

Experimental

We were able to show, using an experimental arrangement described elsewhere,[5] that the pI of Pyrex particles suspended in a solution of trypsin[6] in 0.02 *M* phosphate buffer at 25° was about 7, as reported by Northrop and Kunitz. In 0.02 *M* acetate buffer the mobility remained positive even when the *p*H was increased to 6.7. This latter finding, in which our results are at variance with those of Northrop and Kunitz, was not investigated further.

For purposes of comparison with the moving boundary data of Bier and Nord, mobility measurements were made using Pyrex particles suspended in trypsin solutions in Michaelis buffer[7] with added $CaCl_2$, 0.03 *M*.[8] These results, together with the moving boundary mobility values for the inactive component obtained by Bier and Nord and in our laboratory, are reported in Fig. 1. The moving boundary data for the active component are not included, but the results of our experiment were in accord with those of Bier and Nord.

In addition to the experiments reported in Fig. 1, measurements were made at *p*H 12.5 with a Pyrex–trypsin system in 0.04 *M* glycine–NaOH buffer with added $CaCl_2$, 0.03 *M*. The average mobility, measured at 6° and converted to 0°, was -4.6×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$; Bier and Nord found -4.85×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$ for the inactive component in a moving boundary system under otherwise similar conditions.

As this inactive material, according to moving boundary analysis, comprised only about 8% of the total at *p*H 7.4 (and therefore probably considerably less at lower *p*H values), an attempt was made to remove it from a trypsin solution in acidified water (*p*H 2–3) by contact with an excess of Pyrex particles. When as much as 65% of the ultraviolet absorbing material had been so removed from solution, fresh Pyrex particles suspended in the treated trypsin solution after adjustment to *p*H 8.6 had about the same velocity of migration in the negative direction as particles suspended in a fresh solution of trypsin under the same conditions. On the other hand, active material would have migrated with a large velocity in the positive direction at this *p*H. In another experiment it was found that the loss of 40% of the material coincided with the disappearance of 40% of the enzymatic activity from the solution. The lost activity was not associated with the centrifuged Pyrex particles, which showed only a small degree of activity corresponding to the amount of interstitial solution.

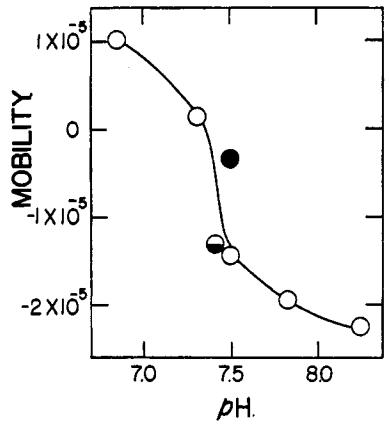

Fig. 1.—Electrophoretic mobilities of components of trypsin and of solid particles immersed in solutions of trypsin: open circles, Pyrex particles immersed in trypsin solutions; Michaelis buffer[7] with added $CaCl_2$, 0.03 *M*; half shaded circle, secondary component in trypsin solutions, moving boundary measurement in same buffer as above; solid circle, data of Bier and Nord[4] for secondary (inactive) component in trypsin solutions, moving boundary measurement in "0.04 *M* barbiturate, 0.03 *M* $CaCl_2$." Measurements by microscope method were made at 6° and converted to 0°; mobility unit: cm.^{2} volt^{-1} sec.$^{-1}$. The moving boundary measurements were made by J. H. Convey of this laboratory.

Discussion

It seems evident that there is an inactive form of trypsin, or an inactive decomposition product, differing electrophoretically from the active enzyme, which is formed at the solid–aqueous solution interface in quantities related to the interfacial area presented. No evidence as to the reversibility of the reaction has been obtained. The coincidence between the mobilities of the coated Pyrex particles and the values given by Bier and Nord for an inactive component suggests that we may be dealing with a single substance, produced relatively slowly in solution, but at an increased rate at the solid–liquid interface.

The results presented here show that considerable restraint should be exercised in interpreting apparent changes in the electrokinetic properties of substances when adsorbed. Unwarranted conclusions may be drawn unless adequate study has been devoted to the possible preferential adsorption of contaminants.

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(2) R. M. Herriott, V. Desreux and J. H. Northrop, *J. Gen. Physiol.*, **23**, 439 (1940).

(3) J. H. Northrop and M. Kunitz, *ibid.*, **16**, 295 (1932).

(4) M. Bier and F. F. Nord, *Arch. Biochem. Biophys.*, **33**, 320 (1951).

(5) R. S. Hartman, J. B. Bateman and M. A. Lauffer, *ibid.*, **39**, 56 (1952).

(6) Crystalline trypsin was purchased from the Worthington Biochemical Laboratory, Freehold, N. J. The specific enzyme activity of this sample was 3.3 ± 0.1 $[T.U.]^{cas}$ per γ, assayed by the optical density method of Kunitz, *J. Gen. Physiol.*, **30**, 291 (1947).

(7) L. Michaelis, *Biochem. Z.*, **234**, 139 (1931).

(8) Bier and Nord refer to the paper by Michaelis[7] in describing the buffer used, but it is not clear whether this buffer had the exact composition given by Michaelis (0.0268 *M* acetate, 0.0286 *M* sodium diethylbarbiturate and 0.0114 *M* NaCl, before adjustment with HCl). Tables IV and V describe the buffer as "0.04 *M* barbiturate."