Further Studies on the Lyo and Desmo Components of Several Hydrolytic Enzymes and Their Histochemical Significance*

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ABSTRACT

This report describes additional studies of the lyo and desmo components of esterase, alkaline phosphatase, acid phosphatase, leucine aminopeptidase, and β-glucuronidase. The techniques used have already been reported (7). Enzyme diffusion occurs to different degrees in different fixatives, and varies somewhat with each enzyme. Loss of enzymatic activity during fixation occurs as a result of both inactivation due to the chemical reaction of the fixative with the enzymic protein, and diffusion of the lyo component into the fixative. The amount of diffusion into formalin can be reduced by the addition of salts, sucrose, or methocel. The pH of the aqueous medium significantly influences the removal of the lyo fraction from the tissue section. A striking similarity can be noted in the proportions of each fraction of enzyme present in the kidney of the rat, dog, and man. The procedure of fixation and paraffin embedding of tissue blocks does not wholly prevent the diffusion of the lyo component from the tissue sections when they are subsequently immersed in the aqueous incubation medium.

In the early attempts to isolate pure enzyme fractions from tissues, it was observed that with many enzymes part of the activity could be readily extracted into the solvents, while the remainder appeared to be bound to the cellular debris. This finding resulted in the introduction of the terms *lyo-* and *desmoenzyme*, the former indicating the soluble component, and the latter the bound fraction (1). Although this differentiation was referred to commonly in enzymological investigations, its significance for histochemical methodology was largely neglected. Except for the admonition of Lison (2) that one can demonstrate only the desmo component of alkaline phosphatase in tissue sections, little discussion of this matter is to be found in the histochemical literature until quite recently.

During the early stages of the development of histochemical methods for enzymology, the mere appearance of a brilliant pigment which remained localized in tissue sections was heralded with a certain satisfaction, and did indeed represent some accomplishment. Subsequent efforts to improve the preciseness of localization were directed toward finding (*a*) modifications of the substrate that would give a more insoluble or substantive reaction product, (*b*) alterations in the incubation media or the capturing reagent that would achieve a more rapid capture of the reaction product, and (*c*) variations in the capturing agent that would provide a pigment with more favorable optical properties. These developments led to striking improvements in the localization of some enzymes (3–6). Obviously, the degree of precision possible was also related to the fixed position of the enzyme in the cell. For a firmly bound enzyme, such as succinic dehydrogenase, the extent of intracellular localization would be determined primarily by the properties of the chemical reaction and reaction products. On the other hand, the limitations imposed by a highly diffusible enzyme would be great no matter how

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ideal the chemical reagents and in many cases the results would be less than satisfactory. A more detailed discussion of these factors and their influence on the localization of enzymic sites has been presented elsewhere (3, 5).

In a preceding publication, a technique was described whereby an estimation could be made of the lyo and desmo components of enzymes in tissue sections under conditions used in histochemistry (7). Significant differences were found in the amounts of each fraction of several hydrolytic enzymes. The duration of incubation, temperature, pH, and salt concentration of the solution were observed to influence the diffusion of lyoenzyme from the tissues. Enzyme loss by diffusion was noted even after fixation of the sections. The work reported in this paper represents an extension of this study with the aim of elucidating additional facts concerning the lyo and desmo fractions of esterase, alkaline and acid phosphatase, leucine aminopeptidase, and β-glucuronidase. The significance of these observations for the intracellular localization of enzymes by histochemical techniques will be discussed.

Materials and Methods

Preparation of the Tissue Sections.—The technique used was similar to that described in the previous report (7). It should be emphasized that for comparative quantitative work of this type, it is important that the amounts of enzyme used for each condition be as nearly equal as possible. This was achieved by attention to two details. First, the blocks were taken by transversely sectioning the kidneys,[1] avoiding the superior and inferior poles, and including the medullary and cortical portions, while trimming off the hilar structures. The blocks were cut with parallel sides to provide a uniform section area throughout. Secondly, to eliminate possible variations in enzyme activity at different levels in the block, the sections were alternated for the various experiments. Furthermore, at least 8 sections were used for each experiment. Thus, if a control and four different sets of conditions were to be tested, 40 sections were cut in a consecutive manner, with sections 1, 6, 11, 16, etc., as the control, sections 2, 7, 12, 17, etc., as the test slides. That the experimental error was well below 10 per cent was verified by repeated findings of close agreement when a control group of sections taken at the beginning of the cutting procedure was compared with a group taken at the end.

For the preparation of paraffin-embedded sections, blocks of rat kidney were fixed in cold acetone (3 changes) for a 24 hour period, then passed through a 1:1 mixture of acetone and benzene for 1 hour, two changes of pure benzene for 1 hour each, then followed by melted paraffin (56°C.) for 1 hour. Sections 10 microns thick were cut on the day of the experiment.

Treatment of the Sections.—In placing the slides in the various solutions, careful handling was necessary to prevent the sections from floating away in the medium. Drying the sections either in the cryostat or at room temperature tended to promote this undesirable occurrence. The sections were used as soon as possible after being cut and thawed.

The mounted sections were placed in the test solution as described elsewhere (7), then removed, and homogenized carefully in a motor-driven ground-glass homogenizer until there was no gross evidence of particulate material. A 2 ml. volume of solution was used for each fresh section tested, while 1 ml. was used for each paraffin-embedded section. The solution in which the sections were soaked contained the lyo enzyme (L), and the homogenate contained the desmo component (D). The control sections were homogenized without previous soaking, and contained both the lyo- and desmoenzyme fractions (T). Thus, there were three enzyme solutions of equal volumes, two as homogenates (T and D) and one as a clear aqueous solution (L). These enzyme preparations were of sufficient volume to allow simultaneous determinations of all 5 hydrolytic enzymes studied.

Determination of Enzymatic Activity.—The incubation solutions were prepared according to the method described in the previous paper (7). The substrates used were β-naphthyl acetate for esterase, β-naphthyl phosphate for both alkaline and acid phosphatases, L-leucyl-β-naphthylamide for leucine aminopeptidase, and 6-bromo-β-naphthyl-β-D-glucuronide for β-glucuronidase. The amounts of homogenate used, the temperature, and duration of incubations for the enzyme determinations are given in Table I of the preceding paper (7).

EXPERIMENTAL

Diffusion of Enzymes into Formalin.—Following publication of the report that exposure of tissue blocks to cold formalin (10 per cent) did not seriously injure the activities of several hydrolytic enzymes (8), the procedure of formalin fixation was employed in many histochemical investigations because it provided for greater ease in preparing sections, for improved preservation of cytologic structure and, presumably, for better immobilization of the enzyme within the cell.

[1] The kidney was selected as the organ for study because of its high content of the five hydrolytic enzymes tested. Although some other tissues had a more uniform cellular content (liver, prostate), they were not sufficiently active for all the enzymes studied.

The amount of enzymatic activity lost has been considered to result from inactivation due to a chemical reaction between the formalin and certain functional groups of the enzymic protein. That this factor alone does not account for the decreased activity is suggested by the finding that diffusion of lyoenzyme into distilled water occurs even after fixation of an 8 μ section in formalin for 30 minutes. The next logical consideration appeared to be the matter of how much enzyme is lost by chemical inactivation and how much by diffusion of the lyo component into the fixative.

The design of experimental conditions to answer this question proved somewhat difficult. Although it was possible to measure inactivation of enzyme by formalin, the exposure was unavoidably longer than desired. On the other hand, it was not possible to measure diffusion loss under conditions free of the influence of inactivation. The conditions that appeared to be best suited for the study of this question were as follows: Two groups of sections were homogenized, one in water and one in formalin, while two other groups were immersed in water or formalin for 1 hour at 4°C., and then removed and homogenized in water or formalin respectively. The first two homogenates contained the total enzymatic activity in water and after exposure to formalin. The latter two groups represent the desmo activity remaining after the water or formalin exposure. Because the enzymatic determinations could not be carried out in the presence of formalin, all four homogenates were dialyzed through Visking cellulose casing from 5 to 17 hours in cold running water before the measurements were made. The solutions were all brought to equal volumes by the addition of distilled water and 2 ml. of a salt solution containing NaCl and KCl (3 gm./liter each), $CaCl_2$ (200 mg./liter), and $MgCl_2$ (40 mg./liter). The salts were added to restore the loss through dialysis, and to aid in resolubilizing the enzymic protein. The results shown in Table I represent the averages of three experiments.

The enzymatic activities remaining after the rather prolonged exposure to formalin are seen in column 3 (Table I). Most of the enzymes are inactivated to a significant degree, esterase being least affected. The values in column 4 represent the enzymatic activities remaining in the tissue sections after immersion in formalin for 1 hour, removal, homogenization in fresh formalin, and dialysis. Since these values reflect both the diffusion and the inactivation losses, it was necessary to correct for the latter. This is shown in column 5 in which, for example, the 25 per cent of esterase activity is divided by 63 per cent, which represents that amount of activity remaining after the formalin exposure, and the corrected value of 40 per cent activity is obtained for the desmo component. This corrected figure indicates the per cent of desmoesterase in the section if there had been no inactivation by the fixative. The same corrections were made for the other enzymes. It may be seen that the corrected desmo activities for both phosphatases are about equal to the total activity, indicating that there was no loss of the lyo component of those enzymes into the formalin during the 1 hour exposure. Thus, all of the inactivation of the phosphatases was due to the chemical reaction between the formalin and the

TABLE I

*Effect of Formalin on Enzyme Activity and on Diffusion of Enzyme from Tissue Sections**

Enzyme	No fixation		Formalin		
	Total	Desmo	Total	Desmo	Corrected desmo
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Esterase	100	30	63	25	40
Alkaline phosphatase	100	61	29	34	117
Acid phosphatase	100	48	12	12	100
Leucine aminopeptidase	100	65	20	16	80
β-Glucuronidase	100	5	6	4	66
Column No.	1	2	3	4	5

* Sections of rat kidney were homogenized in water (column 1) and formalin (column 3) while others were immersed in water or formalin for 60 minutes at 4°C., removed, and then homogenized in water (column 2) or formalin (column 4). All four homogenates were dialyzed against cold water for 5 to 17 hours, made up to equal volumes and the enzymatic activities determined. The figures are averages of three experiments. All the values are compared with the total activity in the water sections, except column 5. Here the desmo values are corrected for the loss of enzymatic activity due to the exposure to formalin by dividing the numbers in column 4 by those in column 3 and multiplying by 100. Therefore, the corrected desmo values represent the amount of activity that would be measured in the tissue sections if no inhibition due to the formalin had occurred. (See text for interpretation.)

TABLE II

*Comparison of Desmo Activities of Sections Exposed to Water and Various Concentrations of Acetone and Alcohol**

Enzyme	Water	Total (lyo + desmo) activity	Acetone, *per cent*				Alcohol, *per cent*			
			100	75	50	30	100	75	50	30
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Esterase	100	275	243	167	194	99	126	20	76	93
Alkaline phosphatase	100	144	154	131	127	123	86	103	115	116
Acid phosphatase	100	147	130	78	103	100	76	61	47	96
Leucine aminopeptidase	100	129	118	45	46	104	8	14	8	71
β-Glucuronidase	100	809	838	752	782	455	797	837	751	451

* Rat kidney sections (8 μ thick) were placed in water or the various concentrations of fixatives for 30 minutes at 4°C. The sections were then removed, homogenized in water, and the activities of the desmo components determined. These results represent the averages of 3 experiments. The total activity was determined after homogenization of untreated sections, and is greater than the desmo activity (taken as a 100 per cent) because the lyo component had not been removed. (See text for interpretation.)

enzymic protein during the exposure period. On the other hand, the values for esterase and leucine aminopeptidase are less than 100 per cent but higher than the desmo activities when only water was used (columns 5 and 2). This suggests that the lyo components of these enzymes did diffuse into the formalin, but not as readily as they did into water. The markedly reduced diffusion of β-glucuronidase into formalin is shown by the fact that the corrected desmo activity was 66 per cent as compared to 5 per cent when formalin was not present. However, because of the great degree of inactivation of β-glucuronidase by the formalin, the color density reflecting enzymatic activity was quite low, and therefore, in a range in which error was more significant. Nevertheless, the findings do permit the conclusion that diffusion of the lyo component of β-glucuronidase into formalin is significantly less than occurs in plain water.

Diffusion of Enzymes into Organic Solvents.—For many years, acetone and alcohol were thought to fix tissue by simply replacing the tissue water with a solvent in which the proteins were unable to migrate. The experiments of Seki (9) suggested that two factors were at work; (*a*) the solvent reduced the electric charge on active hydrated groups, and (*b*) it lowered the dielectric constant of the proteins which tended to hold them in place. Folch and Lees (10) reported that some protein molecules are surrounded by a lipoid moiety which is soluble in certain organic solvents. Furthermore, it is known that purification of some enzymes can be accomplished by their extraction into various concentrations of alcohol or acetone. Thus, there appears to be little reason to suppose that these fixatives should prevent all enzyme loss from tissue sections. It has been demonstrated elsewhere (7), that diffusion of the lyo component of several hydrolytic enzymes into water occurs even after acetone and alcohol fixation. The question proposed above with respect to formalin presents itself again. How much is the decrease in enzymatic activity due to inactivation by acetone or alcohol, and how much to diffusion of enzyme into these fixatives?

A. Diffusion into Acetone:

An attempt to answer this question relative to acetone was approached by an experiment somewhat different from that employed with formalin. Since it was not possible to measure the enzyme in the acetone, sections were incubated in acetone and in water for 30 minutes at 4°C., and the activity remaining in the sections was measured. It also seemed advisable to explore the effects of various concentrations of the fixative. The desmo activities in the sections which had been placed in acetone were contrasted with those of sections which had been placed in water—the figure in water was arbitrarily assigned the value of 100 per cent (Table II). The total activity for the several enzymes was determined after homogenization of sections which had not been previously placed in any solution. The values for the total tissue activities are greater than 100 per cent since they represent both the lyo and desmo components. It may be noted that absolute acetone prevents

any significant loss in total activity, while the lyo component does diffuse out at lower concentrations of the fixative. At 30 per cent acetone, the *esterase*, *acid phosphatase*, and *leucine aminopeptidase* activities are equal to those found in the sections exposed to water; while diffusion of the lyo component of *alkaline phosphatase* and *β-glucuronidase* has been markedly slowed even at this low concentration of the fixative. These findings also show that exposure to cold absolute acetone for 30 minutes does not result in any significant inactivation of these enzymes so that losses at lower concentration of acetone must have been due to diffusion. Two interesting findings were that (*a*) *acid phosphatase* appeared to be more soluble in 75 per cent acetone than in the other concentrations tested and (*b*) the solubility of *leucine aminopeptidase* was markedly increased at acetone concentrations of both 50 per cent and 75 per cent to the extent that less than one-half of the desmo component remained in the sections.

B. Diffusion into Alcohol:

The enzymatic activities of sections exposed to absolute ethanol represent the activity remaining after the loss due to diffusion into and inactivation by the fixative. When these activities are compared with those of untreated sections, it may be seen (Table II) that esterase and both phosphatases are about one-half as active as the controls (total activity). β-Glucuronidase is unaffected by the alcohol exposure, while leucine aminopeptidase is almost completely inactivated. The finding that *esterase* activity in sections exposed to absolute alcohol is 26 per cent greater than the desmo activity in water-treated sections suggests that diffusion of the lyo component is retarded to some degree, but exactly how much cannot be stated because the amount of inactivation produced by the fixative is not known. The diffusion of the lyo component of *alkaline phosphatase* in the lower alcohol concentrations (30 and 50 per cent) is less than that in water. The decrease in activity with increasing strengths of alcohol probably represents inactivation of the enzyme by the fixative. However, *acid phosphatase* does not diffuse to any significant degree into the absolute alcohol; inactivation accounts for the reduction in activity of the tissue to about one-half of the total. This is supported by the report of Stafford and Atkinson (14) that tissue blocks (when diffusion is at a minimum) fixed in alcohol for 28 hours retain only 52 per cent of their total acid phosphatase activity. The lyo component of *β-glucuronidase* is markedly retarded in its diffusion by the lower alcohol concentrations and completely immobilized by the stronger solutions. As is the case with acetone, at certain concentrations of alcohol (75 and 50 per cent) there is increased solubilization of esterase and acid phosphatase respectively. That this represents largely solubilization of the enzymes at these specific solvent concentrations, and not inactivation, is supported by the finding of increased activities in those sections exposed to the higher alcohol concentrations. In summary, the lyo components of esterase and alkaline phosphatase are found to diffuse into absolute ethanol, although not as well as they diffuse into water. On the other hand, acid phosphatase and β-glucuronidase do not diffuse into this fixative. No parallel conclusions can be made with regard to leucine aminopeptidase because of the marked inactivation of this enzyme by the fixative.

Influence of Enzyme Diffusion in Formalin by the Addition of Various Reagents.—Table III shows the activities remaining in sections after exposure for 30 minutes at 4°C. to formalin alone and with certain additives. Increasing the concentration of formalin did not prevent enzyme diffusion, and produced a significant degree of inactivation of β-glucuronidase. Greatest esterase activity remained when either sodium acetate or methocel[2] (15 C.P.S.) was added to the fixative. Little or no increase was noted for alkaline phosphatase or leucine aminopeptidase, but with acid phosphatase, the desmo activity was greater when the methocel was present in the formalin. Since it has been shown earlier that no significant diffusion of acid phosphatase occurred in formalin, this finding suggests that the methocel may have in some manner protected the enzymic protein against inactivation by the formalin. The activity of β-glucuronidase was almost doubled by the additions of salts and 0.88 M sucrose, while methocel had an even greater effect, probably due mainly to retardation of lyo diffusion, and partly to protection against formalin inactivation. In addition to the reagents listed in the table, calcium acetate (20 per cent), sodium sulfate (22 per cent), ammonium sulfate (22 per cent), zinc acetate (20 per cent), and chloral hydrate (1 per cent) in 10 per cent formalin were tested. In general, none of these reagents showed any striking retardation of enzyme diffusion from the sections, and in

[2] Methocel is a methylcellulose and may be obtained from the Dow Chemical Company, Midland, Michigan.

TABLE III

*Desmo Activities in Sections of Rat Kidney after Exposure to Formalin Alone and with Various Added Reagents**

Fixative	Esterase	Alkaline phosphatase	Acid phosphatase	Leucine aminopeptidase	β-Glucuronidase
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Formalin (10 per cent)	100	100	100	100	100
Formalin (20 per cent)	106	84	102	87	29
Formalin + NaCl (18 per cent)	114	109	116	116	195
Formalin + NaAc (20 per cent)	156	86	83	103	185
Formalin + sucrose (0.25 M)	96	92	82	101	90
Formalin + sucrose (0.88 M)	116	115	100	103	192
Formalin + methocel (1.5 per cent)	134	94	135	104	266

* The sections were immersed in the various formalin mixtures for 30 minutes at 4°C. They were then removed, briefly rinsed, and homogenized in water. The activity remaining in the tissues was determined with those values for formalin (10 per cent) being taken as 100 per cent activity. The results are averages of six experiments except for the methocel determinations (two experiments).

some there was significant inactivation of the enzymes. The benefit ascribed by Fishman (11) to the addition of chloral hydrate to the fixative for histochemical demonstration of β-glucuronidase, is not due to the prevention of enzyme diffusion. These results suggest that the addition of various reagents to the fixative does decrease the diffusion of some enzymes into the fixative. Further investigation of their possible value in histochemistry seems to be warranted.

Influence of pH on Diffusion of Lyoenzyme.— In the preceding work (7) it was found that the desmo activities of alkaline phosphatase and leucine aminopeptidase were significantly higher in solutions of nearly optimum pH than in distilled water. Since many of these hydrolytic enzymes have significant activity over a rather wide range of hydrogen ion concentration, it seemed advisable to investigate in more detail the influence of pH on lyoenzyme diffusion. Sections were incubated for 1 hour at room temperature in buffers ranging from pH 4 to 11.5, removed, homogenized in distilled water, and the desmo activities compared with the total activities. The buffers used were 0.1 M acetate (pH 4.0 through 6.5) 0.05 M veronal (pH 7.0 through 9.5) and 0.1 M glycine (pH 10 and greater). The averages of three experiments are shown in Fig. 1. The diffusion of the lyo components of esterase and β-glucuronidase was least between pH 5.0 and 7.0 as indicated by the greatest desmo activities at those pH values. Alkaline phosphatase diffusion was minimal over a broad range from pH 5.0 to 10; while acid phosphatase showed greatest desmo activities between pH 5.5 and 7. The desmo activity of leucine aminopeptidase increased progressively to pH 7.0, and remained relatively constant at the higher pH values.

In interpreting these results, it is necessary to consider the influence of salts present in the buffer solutions. The only data available concern the effect of 2 M NaCl on lyo diffusion (7), and this molarity is a great deal more than the molarities of the buffers used. Nevertheless, alkaline phosphatase and leucine aminopeptidase were the only enzymes in which the desmo activity was preserved significantly by the added salts. Therefore, the results described above for esterase, acid phosphatase and β-glucuronidase more likely represent the influence of hydrogen ion concentration than they do any salt effect. One might suspect that pH is more critical than salt effect in reducing diffusion of the lyo component of the remaining two enzymes from the observations that a sharp decrease in desmo activities occurs below pH 5.0 and above pH 10 for alkaline phosphatase, and a progressive decrease occurs below pH 7.0 for leucine aminopeptidase. To prove that inactivation of the enzymes did not occur as a result of exposure to extremes of hydrogen ion concentrations one experiment was devoted to measurement of lyo activities in the buffers and the expected lyo activities were found.

Comparison of Lyo and Desmo Components in Several Animal Species.—Because enzymatic histochemical techniques are applied to a variety of species, a study was undertaken of the lyo- and desmoenzymes in the kidneys of the rat, dog, and man. Sections were immersed in water for 1 hour at room temperature and the lyo and desmo

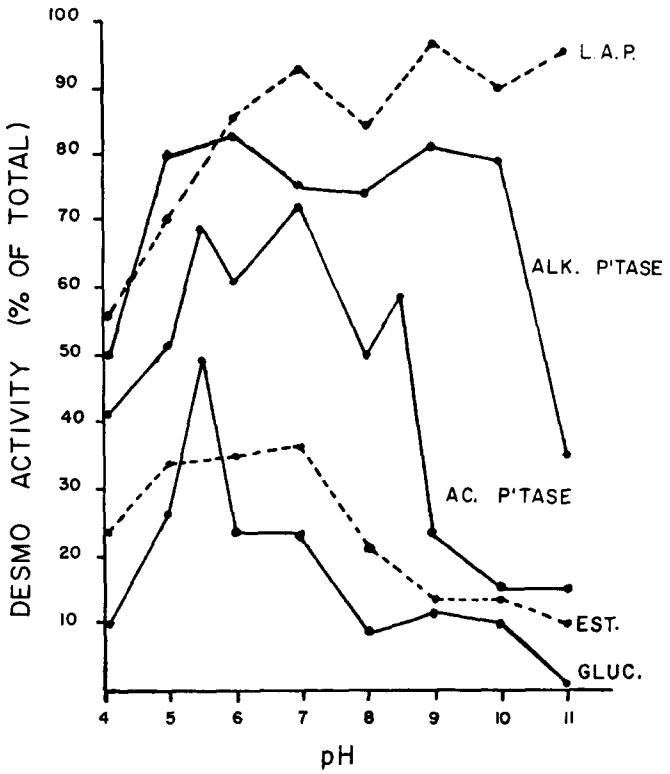

FIG. 1. Sections of rat kidney were exposed for 60 minutes at room temperature to various buffer solutions (acetate, pH 4 through 6.5; veronal, pH 7 through 9.5; and glycine, pH 10 and 11). The sections were removed, homogenized in distilled water, and the desmo fractions measured for enzymatic activity of esterase (EST.), alkaline and acid phosphatases (ALK. and AC. P'TASE), leucine aminopeptidase (L.A.P.), and β-glucuronidase (GLUC.). The percentages were calculated also by determining the total enzymatic activities of kidney sections containing both lyo and desmo fractions.

components measured. The averages of five experiments are shown in Table IV. There was slightly more lyoenzyme of alkaline phosphatase and leucine aminopeptidase in man than in the rat or dog. In general, however, the distribution of the total activities into lyo and desmo fractions was remarkably similar.

Lyo- and Desmoenzymes in Paraffin Sections.—Many histochemical studies are performed on paraffin-embedded sections. Although Cleland (12) has stated that enzyme diffusion does occur in such preparations, the degree of diffusion has not been determined. The averages of 4 experiments on the lyo and desmo fractions of the hydrolytic enzymes studied are given in Table V. Comparison with the lyo components of fresh frozen sections showed no significant differences in esterase diffusion and only slight differences in β-glucuronidase diffusion. However, there were rather striking reductions in the lyo fractions of paraffin-embedded tissue for alkaline phosphatase (17 per cent compared to 39 per cent), for acid phosphatase (26 per cent compared to 52 per cent), and for leucine aminopeptidase (20 per cent compared to 35 per cent). Since it has been shown above that no significant loss of enzyme occurs in acetone, these findings suggest that the procedures of clearing in benzene, exposure to 54°C., and paraffin-embedding effect a partial immobilization of the lyo fractions of several hydrolytic enzymes. Of greater import, however, is the realization that even in paraffin-embedded sections, diffusion of enzyme from the section into the aqueous incubation medium is responsible for a significant loss of activity from the histochemical preparation.

Influence of Various Agents on the Activities of the Lyo and Desmo Fractions.—It appeared reasonable to suspect that differences might be noted in the response of the two enzyme fractions to activa-

TABLE IV

*Comparison of Lyo- and Desmoenzymes in the Kidney of the Rat, Dog, and Man**

Species	Enzyme fraction	Esterase	Alkaline phosphatase	Acid phosphatase	Leucine aminopeptidase	β-Glucuronidase
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
Rat	Lyo	64	30	58	26	108
	Desmo	28	56	51	65	12
	Total	92	86	109	91	120
	Corrected lyo	70	35	53	29	90
Dog	Lyo	71	26	46	33	123
	Desmo	47	54	59	59	8
	Total	118	80	105	92	131
	Corrected lyo	60	33	44	37	94
Man	Lyo	78	41	56	39	94
	Desmo	33	32	46	57	24
	Total	111	73	102	96	118
	Corrected lyo	70	56	55	41	80

* Lyo represents the per cent of enzymatic activity in the diffusate. Desmo represents the enzymatic activity remaining in the tissue. That the sum of these two values is slightly more or less than the activity in the total tissue (100 per cent) has been observed previously and an explanation has been suggested (7). The corrected lyo values represent the per cent of lyoenzyme, when the total is considered to be the sum of the observed lyo and desmo activities.

tors or inhibitors. The lyo and desmo components were separated, and incubated with the substrates in the presence of a variety of cations at concentrations of 10^{-3} M (Mg^{++}, Mn^{++}, Ba^{++}, Zn^{++}, Cu^{++}). In addition, sodium taurocholate, sodium fluoride, and potassium cyanide were also studied at 10^{-3} M concentrations. With the five hydrolytic enzymes investigated, these reagents produced all varieties of effect,—activation, inhibition, or no effect. However, the important observation was that with one exception, the lyo and desmo fractions of each enzyme reacted in essentially the same manner with each reagent. The exception noted was with Mg^{++} and alkaline phosphatase. Here, the desmo component was activated more than twice as much as was the lyoenzyme. However, when the lyo and desmo fractions were dialyzed overnight in cold running water, Mg^{++} (10^{-2} M) activated both components of alkaline phosphatase to the same degree. This suggests that the difference in response to Mg^{++} noted in the former experiment resulted from the diffusion of the cation present in the tissue section out into solution with the lyoenzyme, and not that two varieties of alkaline phosphatase are involved.

TABLE V

*Lyo- and Desmoenzymes in Paraffin Sections**

Enzyme fraction	Esterase	Alkaline phosphatase	Acid phosphatase	Leucine aminopeptidase	β-Glucuronidase
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Lyo	83	17	32	23	92
Desmo	51	86	92	94	34
Total	134	103	124	117	126
Corrected lyo	62	17	26	20	73

* Blocks of rat kidney were fixed in acetone, cleared in benzene, and embedded in paraffin at 56°C. Sections (10 μ thick) were deparaffinized by passing them through benzene and acetone, and then immersed in water for 1 hour at room temperature. The lyo and desmo fractions were measured in the usual manner, the percentage values being calculated from the total activity of sections which had not been immersed in water. Since the sum of the lyo and desmo fractions was greater than 100 per cent, it was necessary to correct for the lyo component. These values thus represent the per cent lyoenzyme with reference to the combined measured lyo and desmo fractions. The figures are averages obtained from four experiments.

DISCUSSION

It should be emphasized that the techniques described above for the separation of lyo- and desmoenzyme have very definite limitations (7). Even though careful attention was given to details, by the alternation of the sections used for each variable studied, by the use of many sections for each experiment, and by the performance of several experiments to settle each issue, the values for lyo and desmo activity of some enzymes did not add up to 100 per cent, although the findings were readily reproducible and were qualitatively

valuable. However, the presentation of numerical figures in the tables may imply somewhat more accuracy than is intended. Despite the shortcomings of the method, significant information can be obtained that has a direct bearing on the localization of enzymes in tissue sections by means of chemical reactions.

When a given chemical reaction is being evaluated, or in considering an already perfected one, the question should always be kept in mind of how the mobility of the enzyme influences the degree of intracellular localization obtained. It has been shown that the lyo component of several hydrolytic enzymes is quite variable, that diffusion of enzyme from the tissue section occurs rapidly, and that such diffusion is influenced by the pH of the medium, the temperature, and the presence of salts and non-electrolytes. Even the procedures of fixation or paraffin embedding do not always or completely immobilize the lyo component, when the sections are placed in an aqueous solution. How then can the observations given here be utilized to improve intracellular localization?

The first matter to be considered is the removal of the lyo component, so that the desmo site may be isolated for accurate identification. Since the factors that influence diffusion vary from one enzyme to another, the easiest way to accomplish this should be determined for the particular enzyme under study. For example, it may be seen in Fig. 1 that diffusion of esterase occurs most efficiently at pH 9.0. Thus, if a 20 to 30 minute immersion of a section in a solution at that pH is carried out, it will furnish a preparation in which the desmo site alone can be localized. If fixation is desired to preserve the cytologic structure, the choice of the fixative will influence the time required for the removal of the lyo component on subsequent immersion in aqueous solution. Although different features might have to be considered for each enzyme, it seems likely that a 30 minute immersion of a fresh frozen section in isotonic saline would remove the greatest portion of the lyoenzyme and permit examination of the desmo site for the hydrolytic enzymes studied here.

The second consideration that appears relevant is the possibility of preserving the lyoenzyme in the section. If this were possible, it would provide information as to whether the lyo component is distributed diffusely within the cytoplasm of the cell or in the vicinity of the desmo component. Furthermore, the preservation of the total activity would identify the enzymic site after a much shorter incubation period. This would be of great value in demonstrating enzymes present in low concentrations in certain tissues, or those in which the greater portion of the activity is in the lyo fraction (*e.g.*, glucuronidase and aryl sulfatase). However, the information available at present does not tell us how the lyoenzyme might be completely immobilized. One possibility which should be explored is the technique of freeze-drying, paraffin embedding, and the performance of the visualizing chemical reaction in the paraffin sections. It has been demonstrated previously that the metal precipitation technique for alkaline phosphatase can be performed on the sections without removal of the paraffin (13). Whether this technique accurately localizes the enzymic site, or whether such incubations can be performed for other enzymes, remains to be determined. The azo dye methods would need to be modified to yield pigments that are insoluble in paraffin.

It seems appropriate to emphasize once again that enzyme diffusion occurs during fixation, during the preparation of paraffin-embedded sections, and afterward when the sections are placed in an aqueous solution. The loss of enzymatic activity by exposure to formalin, acetone, or alcohol, which was considered to result from the inactivation of the enzymic protein by the fixative, appears to be due in some instances to the diffusion of enzyme into the fixative. If desired, such loss can be reduced significantly for certain enzymes by the addition of salts, sucrose or methocel to the formalin, and by the proper adjustment of the hydrogen ion concentration of the fixative.

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