THE ENZYMIC DEACETYLATION OF THE CARCINOGEN 2-ACETYLAMINOFLUORENE AND RELATED COMPOUNDS

by

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INTRODUCTION

The *in vivo* hydrolytic removal of the acetyl group of 2-acetylaminofluorene (AAF) after oral administration of this carcinogen to rats has been demonstrated[1]. It has also been shown that a considerable portion of the radioactivity is excreted in the urine and feces after administration of 9-^{14}C-AAF. The rapid elimination of metabolites of AAF in the excreta makes it rather difficult to detect the low concentrations remaining in the tissues of whole animals. For this reason a study of the metabolism of AAF *in vitro* was undertaken. The present paper deals with the first step in the metabolic transformations undergone by AAF, namely the enzymic removal of the acetyl group.

The distribution of radioactivity in the upper gastro-intestinal tract during *in vivo* metabolism experiments[1] was considerably different with the side-chain acetyl-labeled AAF than with the 9-carbon-labeled compound, suggesting that appreciable hydrolysis of the amide might occur there. Deacetylation of AAF did not take place, however, with pepsin or trypsin at their respective pH optima. Very slight hydrolysis was found with 0.2 *N* and 1 *N* hydrochloric acid (0, 0, 0, 0.85, 0.96 and 3.12% and 0.64, 1.4, 2.4, 7.2, 11, and 22%, respectively after 3, 4, 6, 17, 28 and 65 hours at 37°). Further attempts to demonstrate deacetylation in the gastro-intestinal tract will be described in another part of this paper.

Earlier studies (*cf.* the report of BRAY, *et al.*[2] for references) on the enzymic hydrolysis of aromatic acetylamino derivatives dealt with substrates which were relatively water soluble so that an aqueous solution of sufficiently high concentration could be used. On the other hand, the compounds investigated here presented serious difficulties in this respect owing to their low solubility in water.

Preliminary experiments were carried out on aqueous solutions of AAF in a surfactant such as Tween 80*** or Triton X-100†, using homogenates of liver from male Buffalo strain rats as the enzyme source. Deacetylation occurred as evidenced by the formation of 2-aminofluorene (AF). It was noted, however, in attempts to increase the effective concentration of AAF, that less AF was formed in experiments with larger amounts of surfactants. This suggested that the latter had an inhibitory effect on the

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*** Tween 80: Atlas Powder Company.

† Triton X-100: Rohm and Haas Company.

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reaction, and the technique was therefore abandoned. The suspected inhibition by surfactants was demonstrated by additions of measured amounts to digests prepared as outlined below.

The enzymic deacetylation occurred most readily when the solid, finely powdered substrate was homogenized with the tissue to be assayed. AAF, although very slightly soluble in water[3], seems to be more soluble in tissue homogenates, which may be explained by the observations of NEISH[4] that certain purines have a marked solubilizing action on some aromatic compounds. The lipoproteins may also increase the solubility of AAF in homogenates.

MATERIALS

The isomeric 1-, 2-, 3-, and 4-acetylaminofluorene were synthesized in this Laboratory[5–7]. Acetanilide and 2-acetylaminonaphthalene were commercial products. Radioactive 2-acetylaminofluorene-9-^{14}C was obtained from Dr. F. E. RAY[8]. The tumor-bearing rats[9] were donated by Dr. H. P. MORRIS.

Buffer solutions (0.2 molar) were prepared according to Sorensen (borate, pH 8.9) and Clark-Lubs (phosphate, various pH values).

The anesthesized animals were killed by exsanguination through the abdominal aorta. Organs such as the liver were thereby almost completely freed of blood. Where possible, the tissues were perfused with cold saline. Tissue homogenates (0.2 g fresh tissues per ml of buffer) were prepared in a Waring Blendor or in a Potter-Elvehjem type homogenizer with a plastic pestle. Acetone powders were obtained by homogenizing fresh tissue in 10 volumes of chilled acetone (Waring Blendor) followed by filtration and washing with 5 volumes of ice-cold acetone. One gram of liver gave 0.25 to 0.30 grams of dry acetone powder.

METHODS

The digests were prepared by homogenizing the substrate (1 mg/ml) with the tissue homogenates or with the acetone powder (0.1 g per ml of buffer) in an ice-cold Potter-Elvehjem homogenizer for two minutes. Triplicate samples of 1 ml each of the digest were pipetted with a wide-mouth pipette into tubes for each period of incubation. The tubes were incubated at 37° for 2 and 22 hours. The reaction was terminated by the addition of 1 ml of 10% trichloracetic acid solution. After five minutes a mixture of 1 ml of glacial acetic acid and 1 ml of acetone was stirred in and the samples were allowed to stand ten minutes longer. After centrifugation the resulting pellet was extracted again with 3 ml of a 1:1:1 mixture of 10% trichloracetic acid, glacial acid and acetone.

The combined supernatants were assayed for the amount of amine produced by a modification of the procedure of WESTFALL AND MORRIS[10]. A standardizing time study had shown that the color obtained upon coupling increased up to 10 minutes after the addition of nitrite to the amine solution; no further change occurred thereafter for a number of hours. A 20 minute diazotization period was selected for convenient routine work. Two of the triplicate samples were diazotized for 20 minutes in an ice-bath with 1 ml of 0.03 *M* solution of sodium nitrite. One ml of water was added to the third sample to serve as tissue blank. An aliquot (0.1 to 2.0 ml, depending on the color intensity developed) was pipetted into 5 ml of R salt (0.03 *M* in 5 *M* ammonium hydroxide) and the volume of the mixture was adjusted to 7.0 ml with distilled water. The 2 sample tubes were read against the corresponding blank at 525 mμ in a spectrophotometer. A standard curve (optical density *vs.* mg amine) was determined for each of the amines used in this study; a straight line was obtained in every case between an optical density of 0 and 2.0. It was attempted, however, to keep the optical density of the samples below 0.8 by a proper selection of the aliquot of the diazotized solution.

The effect of substrate concentration was determined as follows: 2 mg AAF per ml of homogenate were incorporated into a 1:5 liver homogenate. This mixture was serially diluted with substrate-free homogenate to give digests containing 2000, 1000, 750, 500, 250, 125, 63, and 32 μg/ml AAF. The amine obtained upon incubation for 2 and 22 hours at 37° was determined as described above.

Three concentrations of liver (33, 133, and 194 mg/ml) were incubated with a constant amount of AAF (1 mg/ml) to study the effect of varying enzyme concentration.

The deacetylation was also followed by the use of radioactive 2-acetylaminofluorene-9-^{14}C with the dual purpose of (1) determining whether compounds other than 2-aminofluorene (AF) were formed, and (2) investigating the possible binding of radioactivity to tissue protein[11,12]. Liver acetone powders were used exclusively as enzyme source since preliminary experiments showed that the

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fat contained in homogenates interfered with the subsequent chromatographic separations described below.

At the end of the incubation period an aliquot was withdrawn from each digest for the colorimetric determination of AF produced as described above. Then the reaction was stopped by heating for three minutes on a steam bath followed by the addition of 4 volumes of absolute ethanol. After centrifugation the pellet was extracted twice with 95% ethanol. The combined supernatants were taken to dryness in a nitrogen atmosphere. The residue was taken up in 1.0 ml of absolute ethanol. Suitable aliquots of this solution and radioactive AAF and AF standards were chromatographed on Schleicher and Schull No. 598 paper (ascending technique) using 3 solvent systems: A. *sec*-butanol (300 ml), 3% ammonium hydroxide (100 ml); B. cyclohexane (320 ml), *tert*-butanol (80 ml), glacial acetic acid (40 ml), and water 5.6 ml; C. cyclohexane (400 ml), pyridine (50 ml), and 94% ethanol (50 ml). The chromatograms were exposed to Kodak "No-screen" X-ray film to reveal thepo sition of the radioactive materials. Solvent C afforded the best separation of AAF and AF. Subsequently each band in a chromatogram with solvent C was eluted separately and counted in a windowless counter. The percentage conversion was calculated from cpm AF × 100 (cpm AF + cpm AAF).

RESULTS

Enzyme properties

Initial experiments were designed to provide fundamental information on the properties of the deactylating enzyme using rat liver homogenates (BRAY *et al.*). The effect of pH on the deacetylation reaction was investigated between pH range of 3.2 to 10. No hydrolysis occurred below pH 5 or above 9.5. The activity was fairly constant between pH 7.8 and 9.5, but exhibited a small peak at pH 8.9. A 0.2 *M* borate buffer, pH 8.9 was therefore selected for routine use.

Most of the activity remained in the pellet after centrifugation of a homogenate at 120,000 *g* for one hour on a Spinco preparative centrifuge. Five times more AF was produced by a pellet suspension than by an equivalent volume of supernatant.

The enzyme content of liver homogenates, as measured by the amount of AF produced, was subject to some fluctuation in various runs. Values of AF ranging from 290 μg/ml to 530 μg/ml, and averaging 450 μg/ml were obtained in a 22 hour incubation period. Since the same homogenate of a number of pooled livers was used in our comparative studies, the data reported here are self-consistent even though some variation occurred from experiment to experiment.

The enzyme was rather stable; a homogenate frozen at —10° retained 100 and 50% of its activity after 24 hours and 30 days, respectively. Incubation for 0.5 hour at 45 and 50° did not affect the activity, but at 55° one-half of the enzyme activity was lost. Acetone powders were less active than complete homogenates on the basis of the weight of tissues or extracts adopted in this work. The powder was nevertheless a convenient form of storing the material since its activity was maintained for at least two weeks at 4° C.

Incubation at 25° decreased the amount of AF produced approximately 50% when compared to incubation at 37°. In 2 and 22 hours, respectively, 150 and 300 μg AF were obtained at 25°, while 300 and 490 μg AF were produced at 37°.

The data in Table I show the effect of increasing substrate concentration under otherwise constant conditions on the amount of AF obtained. The deacetylation was substantially independent of substrate concentration above 0.5 mg/ml AAF. The data presented on a percent conversion basis indicate that probably not all of the added solid substrate participates in the reaction. In this paper the results are therefore expressed as micrograms of amine produced per ml of digest with a substrate concentration of 1 mg/ml of digest.

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The amount of AF produced was proportional to the liver concentration (Table II). For routine work 1:5 homogenate of tissues (0.2 g/ml) and 1:10 suspensions of acetone powders (0.1 g/ml) were selected as a compromise between maximum activity and ease in transfer by pipetting.

TABLE I

EFFECT OF SUBSTRATE CONCENTRATION ON AF PRODUCED*

AAF Concentration μg/ml	AF after incubation of			
	2 hours		22 hours	
	μg/ml	%	μg/ml	%
32	18	56	20	63
62	24	38	33	50
125	45	35	54	43
250	68	27	51	21
500	100	20	130	26
750	100	13	160	20
1000	110	11	150	14
2000	110	5	195	10

* Liver homogenate containing 0.133 g wet weight of liver per ml.

No reaction occurred in control experiments (1) in the absence of substrate, (2) in the absence of enzyme source, and (3) with enzyme source inactivated by heating 3 minutes to 100°. Added AF was recovered within the limits of error of the procedure.

Ethyl alcohol depressed the enzymic activity of liver homogenates at levels above 1% (Table III). This solvent was therefore avoided as a vehicle for the substrate.

TABLE II

EFFECT OF LIVER CONCENTRATION ON AF PRODUCED*

Liver concentration g/ml	μg/ml AF after incubation of	
	2 hours	22 hours
0.033	23	43
0.133	90	190
0.194	150	370

* AAF substrate concentration 1 mg/ml.

Enzyme distribution and substrate specificity

Liver was the most active tissue, of those surveyed (Table IV), in its capacity to deacetylate AAF, 2-acetylaminonaphthalene and acetanilide. The enzymic activity of livers of Buffalo strain rats and Irish rats, and of *C* strain mice was of the same order of magnitude, while that of *A* strain mice was somewhat higher.

Incorporation of Tween 80 or Triton X-100 to give digests containing 2% of the surfactant depressed the enzymic activity considerably.

Riboflavin deficiency (8 weeks old Buffalo strain rats maintained on a riboflavin deficient diet for 9 weeks exhibited the usual deficiency symptoms) had no effect on the deacetylase activity.

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The deacetylase activity was undiminished when the reaction was carried out in a closed vessel in a nitrogen atmosphere. This can be expected if the reaction involves only a simple hydrolytic mechanism. Brain showed considerable activity toward AAF, but no or little effect on the other two substrates. The Birmingham group[2] had reported a lack of activity of brain homogenates toward acetanilide which was thus confirmed. Blood serum and testes appear devoid of enzymes capable of hydrolysing AAF. On the other hand, kidney, heart, lung, and spleen exhibited some activity.

TABLE III

EFFECT OF ETHANOL ON AF PRODUCED*

Ethanol μl/ml	*μg/ml AF after incubation of*	
	2 hours	*22 hours*
0	110	290
5	87	260
10	52	240
25	34	160
50	26	150

* Liver (0.2 g/ml) homogenate and substrate AAF (1 mg/ml).

TABLE IV

DEACETYLATION OF 2-ACETYLAMINOFLUORENE, ACETANILIDE AND 2-ACETYLAMINONAPHTHALENE BY VARIOUS TISSUE HOMOGENATES*

Tissue	*Concentration (μg/ml)*					
	AF		*Aniline*		*2-Aminonaphthalene*	
	2 hours	*22 hours*	*2 hours*	*22 hours*	*2 hours*	*22 hours*
Brain	100	260	0	15	5	25
Liver	170	490	460	550	350	820
Liver + 2% Triton X-100	11	90				
Liver + 2% Tween 80	18	80				
Liver powder	55	330				
Liver, riboflavin deficient rat	170	400				
Liver, Strain *C* mice	260	460				
Liver, Strain *A* mice	340	570				
Kidney	85	220				
Heart	100	170				
Lungs	50	90				
Spleen	50	100				

* All tissues from Buffalo or Irish strain rats, except as noted, at a concentration of 0.2 g/ml of digest.

The activity of the livers of rats bearing transplantable tumors[9] was not depressed from normal levels (Table V). However, the Morris hepatoma 3683[13] showed a smaller hydrolytic capacity than liver. The other two tumors, 1643 and 2226, had some activity, but the results were not compared to the corresponding tissues, the mammary gland and harderian gland, respectively, since these tissues were not examined in this study.

The sections of the gastro-intestinal tract (stomach, small and large intestine,

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cecum including contents) were investigated in both fasting and normally fed rats. The homogenates were prepared in the Waring blendor and the substrate was incorporated in a Potter-Elvehjem homogenizer. The stomach digests were made at pH 2 as well as at the standard pH 8.9. However, no evidence of deacetylation was found with the stomach, large intestine, and cecum. The small intestine exhibited a low order of activity, producing 15–21 μg/ml AF in 22 hours.

TABLE V

DEACETYLASE ACTIVITY OF TUMORS AND OF THE LIVERS OF THE TUMOR-BEARING RATS*

Tissue	*μg/ml AF*	
	2 hours	*22 hours*
Hepatoma 3683	54	93
Liver	170	470
Tumor 1643	38	110
Liver	180	500
Tumor 2226	27	77
Liver	160	430

* Standard conditions: Tissue homogenate containing 0.2 g/ml of tissue and substrate concentration of 1 mg/ml.

The enzymic susceptibility of the isomeric acetylaminofluorenes and 2-diacetylaminofluorene to hydrolysis by the enzyme in rat liver homogenates is recorded in Table VI. It is to be noted that the 2-diacetyl derivative, 2-AAF and 4-AAF are acted upon to almost the same extent. The two acetyl groups of the first compound are removed at least as readily as the single acetyl of 2-AAF. The 1- and 3-isomers were more resistant substrates.

TABLE VI

DEACETYLATION OF ISOMERIC AMINOFLUORENES BY RAT LIVER HOMOGENATES*

Substrate 1 mg/ml digest	*μg/ml amine*	
	2 hours	*22 hours*
1-Acetylaminofluorene	26	120
2-Acetylaminofluorene	170	470
2-Diacetylaminofluorene	230	480
3-Acetylaminofluorene	58	120
4-Acetylaminofluorene	210	440

* 0.2 g/ml of digest.

In this connection the enzymic activity of liver, kidney and brain homogenates of male and female $A \times C$ strain rats toward 2-diacetylaminofluorene was studied. No sex-linked difference in the hydrolytic capability was found in any of these tissues even though the livers of the male animals are considerably more susceptible to carcinogenesis by this compound than the livers of female rats[14].

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Radioactive experiments

The radioautographs of the chromatograms showed only the bands corresponding to AF and AAF. No spots were apparent at the positions corresponding to the other known metabolites of AAF. In the enzyme system used under the experimental conditions described the deacetylation reaction was thus isolated with the exclusion of the many other biological transformations to which AAF is susceptible.

It will be noted (Table VII) that the percentage of AF produced as determined colorimetrically in the same digests is in disagreement* with the results calculated from the activity of the AF and AAF bands (R_F values in solvent C:AF 67–80, AAF 35–60). A similar discrepancy between the spectrophotometric assay and radioactive determination of AF, have been reported by PETERS AND GUTMANN[15] although the procedures of these authors appear different in many respects from the methods used in this work.

TABLE VII

PROTEIN-BOUND RADIOACTIVITY; COMPARISON OF AF DETERMINED COLORIMETRICALLY AND ON RADIOACTIVE CHROMATOGRAMS*

		Incubation period		
		0 hour	*2 hours*	*22 hours*
Protein-bound activity	Counts/mg	2.5	4.4	24
	Percent incorporated	0.25	0.91	4.4
2-AF	Percent by radioactivity**	—	2.3	8.5
	Percent by colorimetric method***	—	9	40

* Liver powder (820 mg) incubated with 37° with 3.7 mg 2-AAF-9-^{14}C ($4.34 \cdot 10^5$ counts per minute).
** See text for method.
*** The percentage figures were obtained by assuming that all of the added substrate was available for reaction.

Protein-bound radioactivity

The proteins isolated from the digests at various time intervals were radioactive. The zero hour sample, which was heat-precipitated immediately after the substrate was homogenized into the digest, carried a small amount of radioactivity. The specific activity of the proteins increased with the length of the incubation period. When the acetate buffer soluble proteins were separated prior to the heat inactivation the soluble proteins had a higher specific activity than the insoluble proteins. In a typical 22 hour experiment (363 mg liver powder, 5.25 mg AAF with 615,000 counts) the activity of the soluble proteins was 70 c/mg while that of the insoluble proteins was 41 c/mg.

DISCUSSION

While ordinary solid substrates are not used in enzyme studies some reports involving insoluble materials have appeared recently [16,17]. The behavior of the enzyme system deacetylating AAF is akin to the system hydrolyzing acetanilide described by BRAY *et al.*[2**]. As reported by these authors, brain homogenates showed negligible

* Recent experiments indicate that AF is partially converted, presumably by oxidation, to compounds with different R_F values during paper chromatography. The band corresponding to AF alone is of course less than AF present in the solution before chromatography.
** Experiments using our liver homogenate and a solution of 1.85 mg/ml of acetanilide gave 26 and 63% hydrolysis in 2 and 22 hours, respectively. Similar values were reported by the Birmingham group.

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activity toward acetanilide. It is noteworthy, therefore, that such homogenates exhibited some activity on the larger molecule β-acetylnaphthylamine and considerable activity on the still larger 2-AAF.

The failure of the segments of the gastro-intestinal tract (except for the low activity of the small intestine) to hydrolyse 2-AAF *in vitro* is of some interest. Our earlier data on the *in vivo* metabolism of AAF[1] could suggest that the deacetylation reaction occurred in the upper gastro-intestinal tract, particularly in the stomach[18]. It is obvious that the *in vitro* experimental procedures do not duplicate in all respects the conditions existing in the rat. It seems nevertheless established that the acidity of the stomach is not sufficient *per se* to account for the rate of hydrolysis observed. An enzymic mechanism must thus be involved. However, the peptic enzymes or gastro-intestinal tissue homogenates investigated in the course of this research were incapable of hydrolysing 2-AAF The specificity of the peptic enzymes[19] makes them unlikely agents in the splitting of a molecule such as AAF. On the other hand a recent report[20] by V. EULER *et al.* indicated that the gastro-intestinal tract can metabolize derivatives of *p*-aminoazobenzene *in vitro*. Even if it be assumed that no enzyme capable of deacetylating 2-AAF is available in the gastro-intestinal tract, some mechanism must be present to transport the molecule to the liver, or other organs, where hydrolytic activity has been demonstrated.

The provisional order of decreasing carcinogenic effect of the isomeric acetylaminofluorenes[21] is 2-, 4-, 1-, 3-AAF, where the 2-isomer is considerable more active than the 4-isomer. On the other hand, these two compounds are deactylated to approximately the same extent in the liver. Since 2-AF and 2-AAF have a similar carcinogenic effect, it is possible that the yet untested 4-AF would exhibit a carcinogenicity of the same order as 4-AAF. Predictions with regard to the carcinogenic activity of 1- and 3-AF cannot be made on the basis of the effect shown by the acetyl derivatives, in view of the limited deacetylation undergone by the latter.

The experiments with the carbon 14 labeled 2-AAF have shown that deacetylation is the only reaction occurring under the conditions outlined above. This would be expected since other metabolic reactions such as hydroxylation require a source of energy not provided. It is therefore rather interesting that binding of radioactivity to the proteins took place. The small amount of activity bound in the 0 hour experiment presumably reflects surface phenomena. The increased amount of binding after various incubation periods, however, would indicate that incorporation into the proteins was a factor. The higher specific activity of the soluble proteins as compared to the insoluble proteins corresponds to the results obtained *in vivo*[11].

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SUMMARY

The deacetylase activity of various tissue extracts toward the carcinogen 2-acetylaminofluorene and a number of related substrates has been studied by colorimetric and isotopic analytical methods. Mouse liver, rat liver, rat brain, kidney and heart were decreasingly active. Three transplantable rat tumors exhibited a low order of activity. Decreasing amounts of the substrates listed below were hydrolyzed by rat liver homogenates: Acetanilide, 2-acetylaminonaphthalene, 4-acetylaminofluorene, 2-acetylaminofluorene, 2-diacetylaminofluorene, 1-acetylaminofluorene, and 3-acetylaminofluorene

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Ethyl alcohol and the surface active agents Tween 80, and Triton X-100 depressed the enzyme activity.

The isotopic experiments showed that deacetylation was the only reaction occurring with the procedure employed. Radioactivity was bound to the liver proteins with indications that incorporation had taken place.

RÉSUMÉ

On a étudié l'activité désacétylante d'un nombre d'extraits de tissus envers le composé carcinogène 2-acétylaminofluorene et envers certains substrats alliés par des méthodes analytiques colorimétriques et isotopiques. Le foie de souris, le foie de rat, le cerveau, les reins, et le coeur de rat étaient pourvus d'activité décroissante dans l'ordre cité. Trois tumeurs transplantable du rat montraient une activité réduite. Des quantités décroissantes des substrats suivants furent hydrolysées par des homogénats de foie de rat: Acétanilide, 2-acétylaminonaphthalène, 4-acétylaminofluorène, 2-acétylaminofluorène, 2-diacétylaminofluorène, 1-acetylaminofluorène, 3-acétylaminofluorène. L'alcool éthylique et les substances à activité de surface Tween 80 et Triton X-100 diminuaient l'activité enzymatique.

Les expériences isotopiques ont montré que la désacétylation était la seule réaction qui avait lieu avec la méthode employée. La radioactivité était liée aux protéines du foie avec des indications d'incorporation.

ZUSAMMENFASSUNG

Die Deacetylase-Aktivität von mehreren Gewebe-Extrakten gegenüber dem krebserzeugenden Stoff 2-Acetylaminofluoren und verschiedenen verwandten Substraten wurde mit kolorimetrischen und isotopischen analytischen Methoden studiert. Die Aktivität der folgenden Gewebe nimmt in der angegeben Reihenfolge ab: Ratten-Leber, Ratten-Gehirn, -Nieren und -Herz. Drei transplantable Ratten-Tumoren hatten nur eine geringe Aktivität. Abnehmende Mengen der folgenden Substrate wurden von Ratten-Leber-Homogenaten hydrolysiert: Acetanilid, 2-Acetylaminonaphthalen, 4-Acetylaminofluoren, 2-Acetylaminofluoren, 2-Diacetylaminofluoren, 1-Acetylaminofluoren, und 3-Acetylaminofluoren. Äthylalkohol, Tween 80 und Triton X-100 setzten die enzymatische Aktivität herab.

Die isotopischen Experimente zeigten, dass die Desacetylierung die einzige Reaktion war welche unter unseren Bedingungen stattfand. Die Radioaktivität war an die Leber-Proteine gebunden und es waren Anzeichen dafür vorhanden dass Einbau stattgefunden hatte.

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