tion.[2] More recently, Hilz and Lipmann[3] preliminarily identified active sulfate chromatographically as an adenosine derivative. We wish to report now that active sulfate has been characterized as adenosine-3′-phosphate-5′-phosphosulfate (PAPS). In our system PAPS derives from adenosine-5′-triphosphate + sulfate and its formation must include phosphorylation in 3′-position.

PAPS is assayed by use of the sulfate–nitrophenol transfer enzyme.[2,4,5] For the preparation of the compound, a liver enzyme prepared by a modification of the previously described procedure[3] was used. After heat removal of protein, the solution containing an average of 50 μM. of PAPS was applied to a column of Dowex-1 and all nucleotides except PAPS were removed by elution with 4 N formic acid and 0.3 N ammonium formate.[6] PAPS was then eluted with 5 N formic acid and 1 N ammonium formate. After lyophilization overnight the eluate showed on paper electrophoresis[3] two ultraviolet absorbing spots, the faster moving corresponding to active sulfate as localized earlier. The slower moving substance, located midway between ADP and ATP, was identified as 3′,5′-diphosphoadenosine (PAP). The phosphosulfate link is hydrolyzed completely by 0.1 N hydrochloric acid at 37° in 30 minutes. Therefore, the formic acid procedure causes more or less hydrolysis.

As shown in Table I, the analysis of the eluates gave consistently a ratio of adenosine to phosphate of 1:2, independent of the amount of active sulfate

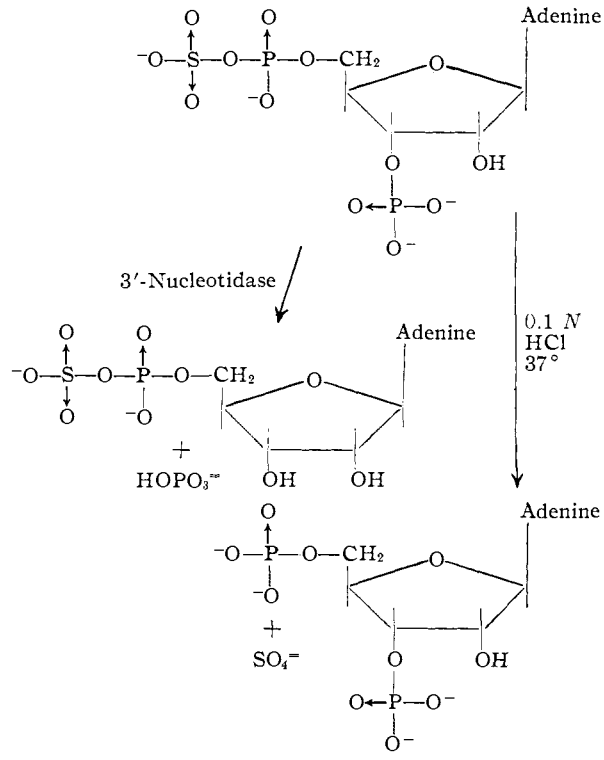
TABLE I

ACTIVE SULFATE FRACTION, ANALYTICAL DATA

Adenosine was determined by absorption at 260 mμ, ribose by the orcinol procedure, and phosphate by the method of Fiske and SubbaRow. Total phosphate was determined after ashing with sulfuric acid. The 12 and 30 minute phosphate was determined by hydrolysis in 1 N HCl at 100°. Phosphate hydrolyzable by the 3′-nucleotidase was determined by the method of Kaplan.[8] We are indebted to Dr. Kaplan for a sample of this enzyme.

Adenosine		1
Ribose		0.95
Phosphate	Total	1.98
	12 minutes	0.53
	30 minutes	1.04
	3′-nucleotidase	0.85
Sulfate, enzymatic		0.2–0.85

present. Acid hydrolysis indicated a phosphate in 2′- or 3′-position on the ribose rather than a pyrophosphate. This was confirmed by the absence of the periodate reaction given by the adenosine-5′-polyphosphates.[7] The second phosphate was eventually further identified as located in 3′-position using Kaplan's specific 3′-nucleotidase.[8] Hydrolysis of S^{35}-marked PAPS with 3′-nucleotidase results in the appearance in the electrochromatogram of a new radioactive spot slightly above ADP which corresponds to adenosine-5′-phosphosulfate (APS); this compound is enzymatically inactive. The degradation of PAPS is formulated as follows:



The position of the sulfate was verified by titration. Hydrolysis of a phosphosulfate should liberate an equivalent of secondary phosphate. For example, mild acid treatment of a preparation containing approximately 5 μM. PAPS gave an increase in titrable material between pH 5 and 8, of 6.0 μM. Further identification was obtained through the confirmation of an earlier suspected reversibility of the reaction: PAPS + nitrophenol $\rightleftarrows$ PAP + nitrophenyl sulfate. The reverse reaction was used for the identification of the desulfo residue. In this manner, 3′,5′-diphosphoadenosine was detected in some commercial preparations of ADP. The sulfate acceptor also was obtained by hydrolysis of coenzyme A, known to contain 3′,5′-diphosphoadenosine.[8]

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DEUTERIUM ISOTOPE RATE EFFECTS AND STERIC INHIBITION OF HYPERCONJUGATION

Sir:

Recently some deuterium isotope effects on the rate of solvolysis of 2,4,4-trimethyl-2-chloropentane (I) have been observed in this laboratory. These results have an important bearing on the following two problems of current interest in reaction mechanism theory: (1) the cause of such isotope rate

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effects in solvolysis reactions[1,2] and (2) the effect of alkyl substitution on hyperconjugation.[3-8]

I-1,1,1-d_3 was made via the corresponding alcohol from 4,4-dimethyl-2-pentanone and CD_3MgI. I-3,3-d_2 was made *via* the following sequence: ethyl pivalate, neopentyl-α-d_2 alcohol, neopentyl-α-d_2 iodide,[9] *t*-butylacetic-α-d_2 acid, ethyl *t*-butylacetate-α-d_2 and 2,4,4-trimethyl-2-pentanol-3,3-d_2. The deuterated chlorides were about ninety per cent. isotopically pure. The solvolysis rate constants were measured conductimetrically in "80%" aqueous ethanol at 25.00°.

SOLVOLYSIS RATE CONSTANTS

Compound	k_1 (10^{-4} sec.$^{-1}$)	Reproducibility (3 or 4 runs)
2,4,4-Trimethyl-2-chloropentane (I)	2.212	±0.015
I-1,1,1-d_3	*1.580*	*±0.010*
I-3,3-d_2	2.04	±0.03

ISOTOPE EFFECT OF DEUTERATION, SOLVOLYSIS IN "80%" ALCOHOL AT 25°

Group deuterated	Compound	k_H/k_D
CH_3-	*t*-Amyl chloride[1b]	1.34
CH_3-	I	1.40
-CH_2-	*t*-Amyl chloride[1b]	1.40
-CH_2-	I	1.08

The important fact to be noted is that the isotope rate effect of α-deuteration is so much smaller for the neopentyl than for the ethyl or methyl groups. Since these isotope rate effects arise from a difference in zero-point energy between the initial and transition states,[1,2] the conclusion that the α-C–H bonds of the neopentyl group are not weakened in the reaction process nearly as much as those of the methyl or ethyl groups seems certain. It follows that the electron loss from the α-C–H bonds of the neopentyl group is also relatively much less. This must be correlated with the fact that the neopentyl group is known to be a poorer electron releaser than the ethyl group in many reactions.[3,4,7]

If this parallelism between the low isotope rate effect of deuteration of the neopentyl group and its low electron releasing ability is accepted then the following explanations which have been offered for the latter phenomena can be discarded as not predicting the correct trends in the isotope rate effects: steric shielding of the reaction center,[10] second order hyperconjugation,[4] inductive effect on hyperconjugation,[7] and steric inhibition of bond contraction.[8] The preferred explanation which apparently best fits the data involves the following points: (1) *hyperconjugative loss of electron density from the α-C–H bonds to the reaction center is necessary for the isotope rate effect* and (2) *a condition for maximum hyperconjugative loss is that the C–H bonds have the proper orientation, nearly parallel, to the electron deficient adjacent orbital*[6] and (3) *that the end methyl groups of the neopentyl group sterically hinder the approach of the methylene group to the proper orientation for maximum hyperconjugation.*

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STEROID 21-HYDROXYLATION BY ADRENAL MICROSOMES AND REDUCED TRIPHOSPHOPYRIDINE NUCLEOTIDE[1]

Sir:

Biological reactions involving liver microsomes include protein[2] and cholesterol[3] syntheses and drug oxidation.[4] The enzymatic step for the 21-hydroxylation of 17α-hydroxyprogesterone has now been shown to occur in beef adrenal microsomes.

Plager and Samuels[5] and Hayano and Dorfman[6] have previously reported ATP and DPN dependent steroid 21-hydroxylation in beef adrenal extracts.

In the present study, beef adrenal fractions were prepared by differential centrifugation as described by Schneider and Hogeboom.[7] A 1:1 homogenate was prepared in 0.25 *M* sucrose, 0.1 *M* phosphate buffer at *p*H 6.8 and 0.04 *M* niacinamide. The microsomal fractions were obtained between 20,000*g* and 105,000*g*, washed and recentrifuged. The 105,000*g* supernatant was further purified by discarding protein precipitated at *p*H 5. Steroids were dissolved in propylene glycol and added to tissue fractions which were then incubated for one hour at 37° with air as the usual gas phase. Incubation mixtures were extracted with methylene chloride, evaporated and the residues partitioned between 95% methanol and hexane. The aqueous methanol was evaporated and the enzymatic conversion of 17α-hydroxyprogesterone to 17,21-dihydroxyprogesterone (Reichstein's Substance S) was assayed by development of the Porter–Silber chromogen[8]. The product was identified as 17α,21-dihydroxyprogesterone by Bush paper chromatography,[9] counter-current distribution and infrared spectroscopy.[10] The conversion of progesterone, 11β-hydroxyprogesterone and 11β,17α-dihydroxy-

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