tized animals after being returned to ground level and sacrificed. When killed at altitude to which the animal was acclimatized, however, brain lactic acid was significantly elevated over ground level control and when killed at higher altitude the brain lactic acid increased still further but was considerably below the level found in intact non-acclimatized animals exposed to the same altitude. It appears that acclimatization changed the usual response to altitude as far as brain lactic acid was concerned, probably by altering the lactic acid precursor level of the blood.

Summary. In this study, adrenalectomy prevented a rise in lactic acid content of brain tissue in hypoxic rats. When various adrenal hormones were injected, the brain lactic acid level of adrenalectomized hypoxic rats was elevated to the same extent as intact hypoxic rats. Animals adapted to a simulated altitude of 18,000 feet did not respond to the same degree as non-adapted animals when both groups were exposed to severe hypoxia.

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Labelling of Antibodies by 5-Dimethylamino-1-Naphthalene Sulfonyl Chloride, Its Effect on Antigen-Antibody Reactions. (23959)

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Histochemical technics using antibodies (Ab) labelled with fluorescent dyes have gained increasing application to immunological problems(1,2). The present paper deals with 5-dimethylamino-1-naphthalene sulfonyl chloride (DNS),† a fluorescent dye recently introduced by Clayton(3). Investigation of the usefulness of this compound for visualization of Ag-Ab reactions was undertaken because of the simple procedure for labelling proteins and the increasing knowledge about the nature of the protein-DNS union(4,5). An enzyme-antienzyme system, yeast alcohol dehydrogenase (ADH)-rabbit antiserum(6,7), was chosen to determine whether labelling of Ab with DNS has any influence on formation of Ag-Ab complex.

Methods. Chemical properties. DNS is a yellow substance which emits yellow fluorescence. It is almost insoluble in water, but readily soluble in organic solvents like ethanol, ether, ethyl acetate, acetone and chloroform. The corresponding sulfonic acid is formed slowly by hydrolysis but the dye can be used for a few months when kept dry and in the cold. The protein-DNS complex, however, is more stable. Labelled proteins can be precipitated with alcohol, acetone and ammonium sulfate without splitting of the protein-DNS bond. DNS and conjugates absorb light in 300-400 mμ with maxima about 320 to 355 mμ according to medium, pH and combination with different proteins. *Conditions for labelling proteins and DNS-protein ratio.* Proteins buffered to pH 7.4-8.0 are readily labelled by mixing with appropriate amount of DNS dissolved in acetone(4) or ethanol (Vinograd)‡. The suspended unbound dye can be removed by centrifugation while dis-

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TABLE I. Binding Capacity of Serum Gamma-Globulin and Albumin for DNS.

Protein	DNS	Quotient: $\frac{\mu M \text{ DNS bound}}{\mu M \text{ protein}}$	
⌜——μM——⌝		γ-globulin	Albumin
.105	3.71	4.1	7.3
.210	"	3.8	7.5
.525	"	3.2	7.0

solved unbound dye may be separated by dialysis or more speedily (together with some dye which may be bound by adsorption) by precipitation of the proteins, as mentioned above, and resolution of washed precipitate. Experiments were performed with purified human gamma globulin (Lederle) and crystallized bovine albumin (Pentex), to measure the quantitative ratio in binding of proteins and DNS. A constant amount of DNS (0.1 ml of a 1% solution in ethanol) was mixed with 16.5, 33.0, and 82.5 mg gamma-globulin and with 7.3, 14.6, and 36.5 mg albumin dissolved in 5 ml phosphate buffer M 0.05, pH 7.4. After 5 minutes at room temperature, samples were immersed in ice bath for 30 minutes. The labelled protein was precipitated with 11.5 ml acetone and samples centrifuged at 5°C for 10 minutes (10,000 x g). The clear yellow supernatant was measured in a spectrophotometer at 366 mμ against dye standards treated in a similar way. The molar ratios of DNS bound to the tested proteins were calculated on the basis of M.W. 156,000 for gamma-globulin and 69,000 for albumin respectively. The observed differences in degree of labelling of albumin and gamma globulin are due to differences in binding sites which appear to be basic groups (most likely primary amino groups) imbedded in lipophilic environment(4,8). From reported data the conclusion can be drawn that all protein molecules present in the experiments are labelled under the stated conditions. The degree of labelling depends on the optimal concentration of DNS. *Reaction rate of Ag-Ab complex formation with labelled Ab.* The use of the ADH-rabbit antiserum system offers a satisfactory method for measurement of Ag-Ab reaction rates. Inhibition of enzyme, as measured by optical test of Warburg(9), corresponds to an early phase of the Ag-Ab combination. Any factors capable of exerting a specific action on this stage of the Ag-Ab reaction should be detectable (a thorough study of the use of this method for measurement of Ag-Ab reactions will be published). Serum of rabbits immunized with crystalline ADH was used as source of Ab. A partly purified globulin fraction of antiserum was obtained by precipitation with 40% saturated ammonium sulfate and dialysis against saline. The globulin fraction and a corresponding globulin fraction from an unimmunized animal were labelled with an excess DNS as described above. Beckman cells were filled with 2.3 ml phosphate buffer 0.06 M, pH 7.4 (containing 0.02 M semicarbazide), 0.1 ml diphosphopyridine nucleotide (c = 15 mg/ml), and 0.1 ml ethanol (50%). After addition of 0.05 ml Ab fraction or normal globulin fraction, both labelled and unlabelled (each containing 0.55 mg protein), the reaction was started with 4 μg ADH, and the increase in optical density at 340 mμ was recorded every 15 seconds for 120 seconds. The relative enzyme activity is expressed by the increase in optical density (D) in stated time interval, while inhibition is expressed in per cent of corresponding activities of tests with Ab and blank without Ab $\left(100 - D_{Ab}/\frac{D_{control}}{100}\right)$.

Results. The increased inhibition in time interval 60-90 sec. compared with that of 45-60 sec. shows progressive formation of Ag-Ab complex. This reaction is completed to about 95% within 5 min. after addition of Ag to Ab. As indicated in Table II, there is practically no difference in reaction rate with

TABLE II. Reaction Rates of Ag-Ab Complex Formation (ADH-anti-ADH System) with Labelled and Unlabelled Ab.

Test	Optical density Δ 45-60 sec.	Δ 60-90 sec.
Normal globulin unlabelled	.063	.115
Ab " "	.038	.066
Inhibition	(39.7%)	(42.7%)
Normal globulin labelled	.063	.115
Ab " "	.040	.067
Inhibition	(36.5%)	(41.8%)

labelled and unlabelled Ab. In another set of experiments, varying concentrations of DNS were added to tubes containing 4.5 mg purified Ab globulin. Enzyme inhibition by Ab was measured against samples containing similarly treated normal globulin fraction. The results are presented in Table III.

Enzymatic tests with the ADH-anti ADH system demonstrate specific response in an Ag-Ab reaction. These are performed in an excess of Ab within the range of about 1 Ag molecule to 50 Ab molecules. An effect of DNS on the enzyme itself could be an interfering factor in this type of experiments, however, unspecific DNS effects do not occur when blank tests are performed in which a normal globulin fraction is treated exactly as the Ab fraction. Labelling of the enzyme (Ag) can be prevented when the free suspended DNS is separated by centrifugation and precipitation of the Ab preparation. The dissolved free DNS has no measurable effect on activity of the enzyme, neither has the DNS bound to other proteins, but ADH is rather markedly inhibited (up to 75%) when labelled directly with excess of DNS. This inhibitory action may be similar to the effect which DNS exerts on chymotrypsin(10). All differences in inhibition of enzyme activity by labelled and unlabelled Ab in Table III are in the range of experimental error. It appears that DNS has no effect on rate of Ag-Ab reaction.

Ag-Ab precipitate formation with labelled Ab. To investigate the influence of DNS labelled Ab on formation of precipitate, Ag-Ab mixtures were incubated at 7°C for 24 hours; sediment and supernatant were analyzed by biuret reaction. The molecular ratio of 1 Ag: 3 Ab molecules in the precipitate was found to characterize the equivalence zone. Experiments were performed with labelled and unlabelled Ab, using different protein concentrations, but keeping Ag-Ab ratio within the equivalence zone. Ab protein concentrations were 4.75, 10.0 and 16.4 mg, and corresponding ADH concentrations 0.046, 0.096 and 0.158 mg respectively. The results are given in Table IV. The analysis provides evidence that labelling of Ab does not influence amount of precipitate formed in the ADH-anti-ADH system.

TABLE III. Reaction Rates of Ag-Ab Complex Formation. Ab labelled with varying DNS concentrations.

DNS added (mg)		.3	.5	.8
% ADH inhibition by Ab (time 45-60 sec.)	36.7	36.7	36.2	40.0

TABLE IV. Total Protein in ADH-Anti-ADH Precipitate Using Labelled and Unlabelled Ab.

	Precipitate (mg protein)		
Labelled Ab	.640	.388	.198
Unlabelled "	.673	.404	.180

Summary and conclusion. Properties of the fluorescent dye DNS for labelling proteins have been described. Investigations of conditions of DNS binding to serum proteins revealed ratios of 3-4 M DNS/M gamma-globulin and about 7 M DNS/M albumin. Dealing with the use of DNS in immunochemistry, experiments have been carried out with the ADH-rabbit antiserum system, measuring enzyme inhibition by the Ab. Labelling of Ab with DNS did not affect rate of formation of Ag-Ab complex and had no influence on quantity of formed precipitate. The reported data suggest that because DNS does not affect the Ag-Ab reaction, it can be successfully applied in histochemical studies.

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