TABLE I

KETONIC HYDRAZONES OF TRIAMINOGUANIDINE

Ketone	Yield,[a] %	M.p., °C.	Formula	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
2-Acetylthiophene	98	217	$C_{19}H_{20}S_3N_6$[b]	53.27	53.14	4.67	4.75[c]
3-Acetylthionaphthalene	93	283	$C_{31}H_{26}S_3N_6$[b]	64.35	64.49	4.50	4.53[d]
Alloxan	94	>300	$C_{13}H_{14}N_{12}O_{12}$[e,f]	29.43	29.3	2.64	2.58
Benzylideneacetone	72	125	$C_{31}H_{35}N_7O_4$[g,h]	65.3	65.85	6.16	6.44
Benzoin	97	190	$C_{45}H_{43}N_7O_8$[g,i]	65.73	65.52	5.47	5.0
Chalcone	75	132	$C_{46}H_{39}N_7O_3$[g]	74.9	74.76	5.29	5.39
Cyclohexanone	56.5	135	$C_{19}H_{27}N_9O_7$[g,k,l]	45.41	45.44	5.57	5.62
Dibenzylacetone	95	227	$C_{52}H_{44}N_6$[g]	83.0	83.0	5.85	5.87
m-Cyanoacetophenone	95	237	$C_{25}H_{23}N_9$[b]	69.28	69.47	4.74	5.0

[a] All yields of crude material. [b] Recrystallized from pyridine and therefore tabulated data are those for the hydrazone free base. [c] Calcd. S, 22.43; found S, 22.49. [d] Calcd. S, 16.61; found S, 16.55. [e] Not recrystallized because of insolubility. [f] This formula is for a trihydrate. [g] Recrystallized from 95% alcohol. [h] This formula is for a monohydrate. [i] This formula is for a dihydrate. [k] Dihydrazone isolated as a picrate. [l] Yellow plates analyzing for 0.5 mole of water of crystallization.

crystals of the dipyrazolemonohydrazone, named above, were almost insoluble in ether, soluble in water and in alcohol. The nitron[4] test proved the absence of nitrate. *Anal.* Calcd. for $C_{16}H_{22}N_6O$: C, 61.1; H, 7.0; N, 26.75. Found: C, 60.8; H, 7.1; N, 26.7.

When the experiment was repeated, under reflux, for half an hour and the product precipitated by pouring into water, a 48% yield of a yellow amorphous powder, which after recrystallization from aqueous ethanol melted at 148° was obtained. Its nature is unknown. Its analysis suggested it to be of empirical formula $C_3H_4N_2$ and it did not form a picrate. *Anal.* Calcd. for $C_3H_4N_2$: C, 52.9; H, 5.8; N, 41.2. Found: C, 52.8; H, 5.7; N, 42.2.

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(4) *Cf.* J. E. Heck, H. Hunt and M. G. Mellon, *Analyst*, **59**, 18 (1934).

COMMUNICATIONS TO THE EDITOR

ENZYMATIC DEGRADATION OF THE CHOLESTEROL SIDE CHAIN IN CELL-FREE PREPARATIONS

Sir:

It has been demonstrated by Bloch, *et al.*,[1] that cholesterol can be converted to cholic acid in intact animals. These earlier studies have recently been extended by Chaikoff and his co-workers[2,3,4] who showed, in both animals and in surviving tissue slices, that the oxidation of the side chain proceeded at a relatively rapid rate while the cyclopentenophenanthrene nucleus was essentially inert. To facilitate a systematic study of the enzyme-catalyzed steps in this oxidation, we have obtained an active cell-free system from mouse liver tissue and report some of its properties below.

Mouse liver mitochondria were prepared by the method of Kielley and Kielley.[5] These preparations were unable to oxidize the C^{26}-carbon atom of 26-C^{14} labeled cholesterol[6] (added as a serum albumin stabilized emulsion) to CO_2, either alone or when supplemented with DPN, nicotinamide, adenylic acid, cytochrome C, magnesium ions and malate. The original sucrose homogenate, on the other hand, oxidized as much as 1.0% of the labeled carbon atom under the incubation conditions described in Table I. Microsome free supernatants, prepared

TABLE I

CHARACTERISTICS OF THE CHOLESTEROL OXIDASE SYSTEM

Individual flasks incubated in air at 37° contained one or more of the following components as indicated: 1.6 cc.[a] washed mitochondria in 0.25 *M* sucrose, M_w; 2.0 cc. homogenate-free of cell-debris and nuclei; 2.0 cc. microsome and mitochondria-free supernatant, S. Other additions were made to each flask unless otherwise indicated, at the following final concentrations: DPN, 0.0005 *M*; AMP, 0.002 *M*; nicotinamide, 0.02 *M*. The final volumes were made to 6.0 cc. with 1:1 0.9% KCl–0.2 *M* potassium phosphate buffer, *p*H 7.4; CO_2 was trapped in the center well in KOH and counted[7] as $BaCO_3$; 0.9 cc. 26-C^{14}-cholesterol was added as a 1% serum albumin stabilized emulsion containing 0.4 mg. cholesterol (42,000 c.p.m.[7])/cc. of saline-phosphate.

Expt.	Time of incubation, min.	Addition	Total c.p.m. in $BaCO_3$
I-1	120	Homogenate	206
2	33	M_w + S	8
3	63	M_w + S	43
4	120	M_w + S	197
5	120	M_w + dialyzed S	157
6	120	M_w + boiled, 3×concentrated S	0
7	120	M_w + S (no AMP)	124
8	120	M_w + S (0.22 cc. cholesterol emulsion)	96
II-1	120	M_w + S (stored at 3° for 24 hr.)	54
2	120	M_w + S (no DPN)	7
3	120	M_w + S (no DPN; TPN 0.0005 *M*)	2

[a] Equivalent to homogenate volume in flask 1.

(1) K. Bloch, B. N. Berg and D. Rittenberg, *J. Biol Chem.*, **149**, 511 (1943).

(2) I. L. Chaikoff, *et al.*, *ibid.*, **194**, 413 (1952).

(3) M. D. Siperstein and I. L. Chaikoff, *ibid.*, **198**, 93 (1952).

(4) J. R. Meier, M. D. Siperstein and I. L. Chaikoff, *ibid.*, **198**, 105 (1952).

(5) W. W. Kielley and R. K. Kielley, *ibid.*, **191**, 485 (1951).

(6) A. I. Ryer, W. H. Gebert and N. M. Murrill, THIS JOURNAL, **72**, 4247 (1950).

(7) C. V. Robinson, *Science*, **112**, 198 (1950).

by centrifugation of homogenates at 120,000 × *g.* for 30 minutes, when added to washed mitochondria, restored the activity to essentially the level originally obtained in the crude system.

Both the crude and partially resolved systems require the addition of diphosphopyridine nucleotide (DPN) and nicotinamide. Triphosphopyridine nucleotide (TPN) does not replace the DPN requirement. The over-all oxidation process shows a partial dependence on the presence of adenylic acid (AMP) although this phenomenon may be related to the stabilizing effect of adenylic acid and adenosinediphosphate on the general metabolic integrity of mitochondria.[5]

As shown in Table I, dialysis of the soluble fraction of the system does not lead to a significant loss in activity, nor can this fraction be replaced by a concentrated, boiled extract of whole liver or liver fractions. The inactivity of either the mitochondrial or the soluble fractions alone, and the demonstration of a considerable lag phase in the appearance of $C^{14}O_2$ suggests, as one possibility, the accumulation of an intermediary compound derived from the side chain carbon atoms which is subsequently oxidized by the terminal oxidizing systems of the mitochondrial elements.

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RECEIVED JANUARY 23, 1953

ENZYMATIC CLEAVAGE OF THE CITROVORUM FACTOR

Sir:

In our studies with soluble enzyme preparations which liberate bound forms of folic acid from liver, we observed that citrovorum factor (CF) added to such preparations disappeared at a rapid rate. We now have obtained from horse liver a protein fraction which, in the presence of *l*-glutamic acid, effectively destroys CF.

The protein fraction was obtained as the 30% saturated (0°) ammonium sulfate precipitate from a cold water extract (0°) of horse liver.

The influence of *l*-glutamic acid on the rate of inactivation of CF by the liver fraction is shown by the data in Table I. The loss of CF activity as measured by both *Leuconostoc citrovorum* and *Streptococcus faecalis* R, is paralleled by a rise in arylamines indicating a cleavage of the pteridine moiety from the *p*-aminobenzoylglutamic acid residue.

TABLE I

RECOVERY OF CF AFTER INCUBATION WITH LIVER PROTEIN FRACTION

Incubated at 37° for 2 hr. in 0.08 *M* Na_2HPO_4; volume, 7 ml.; initial CF = 56 γ.

L-Glutamic acid, *M*	None	0.001	0.002	0.004	0.0075	0.01
CF, γ	51.0	36.8	31.2	26.6	24.2	23.0

The role of *l*-glutamic acid appears to be specific. Other amino acids including *l*-glutamine and also known metabolic products of *l*-glutamic acid do not replace *l*-glutamic acid in this system. It is significant that *p*-aminobenzoic acid inhibits the reaction (31% inhibition with 0.01 *M* concentration of *l*-glutamic and *p*-aminobenzoic acids). While CF is attacked readily by the protein fraction in the presence of *l*-glutamic acid, neither pteroylglutamic acid nor its N-10-formyl derivative is affected.

NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, PUBLIC HEALTH SERVICE, FEDERAL SECURITY AGENCY JOHN C. KERESZTESY MILTON SILVERMAN
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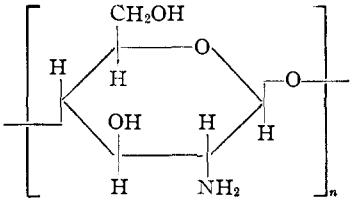
RECEIVED FEBRUARY 20, 1953

DIRECT EVIDENCE OF THE INFLUENCE OF SULFAMIC ACID LINKAGES ON THE ACTIVITY OF HEPARIN-LIKE ANTICOAGULANTS

Sir:

Heparin, the naturally occurring glucosamine-glucuronic acid polysaccharide polysulfate, is characterized by its high anticoagulant activity (U.S.P. Heparin is defined as having not less than 100 International Units per mg.) and by its essentially nontoxic nature (mouse intravenous LD_{50} = 1500–2000 mg./kg.[1]). Extensive work by numerous investigators has indicated that the activity of heparin is dependent, among other things, upon the degree of sulfation of the molecule and recently both Jorpes[2] and Meyer[3] concluded, as earlier considered a possibility[4] and more recently affirmed[5] by Wolfrom, that the amino groups in the molecule are sulfated and demonstrated that hydrolysis of the protected amino linkages resulted in essentially complete inactivation of the material. In order to test the validity of the postulate that the presence of sulfamic acid groups is a major factor essential for the high activity and presumably for the low toxicity of heparin, and also because the stated conclusions were based largely upon indirect evidence, we undertook to obtain direct evidence of the contribution of sulfamic acid linkages to the anticoagulant activity of polysaccharide polysulfate esters of the heparin type.

In this work the polyglucosamine, chitosan



was used as a model substance in sulfation experiments designed for the preparation of products in which the amino and hydroxyl groups were sulfated to varying degrees. Some of the data obtained on some of these products are given in Table I.

Thus, for the first time, there are data which indicate a correlation in agreement with the hypothesis

(1) J. Seifter and A. J. Begany, *Am. J. Med. Sci.*, **216**, 234 (1948).
(2) J. E. Jorpes, H. Boström and V. Mutt, *J. Biol. Chem.*, **183**, 607 (1950).
(3) K. H. Meyer and D. E. Schwartz, *Helv. Chim. Acta*, **33**, 1651 (1950).
(4) M. L. Wolfrom and W. H. McNeely, THIS JOURNAL, **67**, 748 (1945).
(5) M. L. Wolfrom, R. Montgomery, J. V. Karabinos and P. Rathgeb, *ibid.*, **72**, 5796 (1950).