diffusion gradient of this hormone, the cells stream together to form a multicellular unit, which then undergoes further differentiation. Earlier work has indicated that acrasin is stable toward acid and alkali[4,5] and that certain steroids could simulate the hormone to some extent.[6]

We have isolated a sterol with weak acrasin activity in the Shaffer test[3] from *Dictyostelium discoideum*, grown on a complex agar medium in the presence of *E. coli*.[2] At early aggregation, the cells were harvested with cold water, then boiled for 15 minutes to stop enzymatic activity. To each 1–1 liter portion of cell suspension 150 ml. of concentrated hydrochloric acid was added and the material was boiled for 10 minutes. After cooling, the hydrolysate was extracted with CH_2Cl_2. The extract was fractionated according to the procedure previously used for the isolation of fecal steroids.[7] The alcohol fraction, isolated *via* the hemiphthalates, was chromatographed on alumina grade III, and crystalline material was eluted by petroleum ether–benzene (1:1).

No biological activity was detected in the acidic, ketonic, and non-alcoholic fractions and apparently all of the activity was recovered in the crystalline alcohol eluted from alumina.

Since the infrared spectrum of the recrystallized sterol (I) suggested its identity with Δ^{22}-stigmasten-3β-ol (II), an authentic sample of this sterol was synthesized[8] by reduction of $\Delta^{4,22}$-stigmastadien-3-one to Δ^{22}-stigmasten-3-one (III) with lithium in liquid ammonia[9] and further reduction of III to II with $LiAlH_4$.[10] The synthetic II showed the same biological activity and infrared spectrum as I. Oxidation of I with chromic acid[11] gave a product with the same infrared spectrum as III and the acetates of the synthetic II and of the isolated I showed identical infrared spectra. Confirmatory evidence for the identity of I, its oxidation product (dehydro I) and its acetate with II, III and the acetate of I, respectively, is summarized in Table I. Whereas the melting points of the isolated I and its derivatives differed from those of the corresponding authentic samples, presumably owing to polymorphism, they showed no depression upon admixture. The possible relationship of I to the *in vivo* form of acrasin is under investigation. I is not as active as certain crude unhydrolyzed preparations; it may exist naturally as a conjugate, or act synergistically.[15] Details of the isolation procedure and identification will be presented elsewhere.

TABLE I

	M.p.,[12] °C.	$(\alpha)_D$[13]	C, %	H, %
I	156.0–157.0	+ 5.2	83.98	12.09
II	163.0–163.5	+ 5.1		
Literature[14]	159.0	+ 2	84.00	12.15
Dehydro I	173.0–174.0	+20	83.94	11.40
III	170.0–171.0	+22		
Literature[14]	166.0–167.0	+20	84.39	11.74
Acetate of I	156.0	− 4.9	81.29	11.65
Acetate of II	146.0–146.5	− 5.0		
Literature[14]	144.0–144.5	− 6	81.52	11.48

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RECEIVED SEPTEMBER 22, 1959

THE ENZYMATIC CARBOXYLATION OF BUTYRYL COENZYME A

Sir:

The enzymatic ATP-dependent carboxylation of acetyl CoA,[1,2] propionyl CoA,[3–7] and β-methylcrotonyl CoA[8] has been investigated. The present report describes the identification of the product of butyryl CoA carboxylation catalyzed by purified propionyl carboxylase.[6,7]

The carboxylase was prepared from dilute Tris[9] (0.002 *M*, *p*H 7.3) extracts of bovine liver mitochondrial acetone powder. After aging the extract for 20 hours at 30°, the protein precipitating between 45 and 55% saturated ammonium sulfate was dissolved in and dialyzed for 12 hours against 0.002 *M* Tris, *p*H 7.3. Enzyme prepared in this manner had a specific activity of 22 carboxylase units (Flavin, *et al.*[4]) per mg. of protein.

The relative rates of carboxylation of acetyl CoA, propionyl CoA and butyryl CoA catalyzed by ammonium sulfate–purified propionyl carboxylase are summarized in Table I. It is apparent that butyryl CoA and acetyl CoA are carboxylated at significant rates although the rate is much greater with propionyl CoA.

The reaction product of butyryl CoA carboxylation was investigated. Eight units of the purified carboxylase were incubated for 45 minutes at 37° with 200 μmoles of Tris, *p*H 8.5, 8 μmoles of ATP, 8 μmoles of $MgCl_2$, 10 μmoles of glutathione, 2 μmoles of butyryl CoA and 2.5 μmoles (5 μc.) of $KHC^{14}O_3$ in a total volume of 3.0 ml. Following a 45-minute incubation at 37°, 1.0 ml. of 4*N* sodium hydroxide was added; 30 minutes later the mixture was acidified with hydrochloric acid and continuously extracted with diethyl ether for 24

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TABLE I

In addition to 1 μmole of the appropriate acyl CoA derivative each tube contained (in μmoles): Tris, pH 8.5, 100; ATP, 4; $MgCl_2$, 4; glutathione, 5; $KHC^{14}O_3$ (specific activity, 47,000 c.p.m. per μmole), 15 and enzyme. 1.02 and 0.20 units of purified carboxylase (see text) were added to tubes containing acetyl- and propionyl-CoA, respectively. Final volume was 1.5 ml.; 20-minute incubation at 37°.

Additions	$HC^{14}O_3^-$ fixed per hour per mg. of protein[4] (μMoles)
None	0.0
Acetyl CoA	0.3
	0.4
Propionyl CoA	35.2
	33.3
Butyryl CoA	1.7
	1.6

hours. The concentrated ether extract was chromatographed (ascending) on Whatman No. 3MM paper using the isoamyl alcohol saturated with 4 N formic acid solvent system.[3] The single radioactive spot observed (R_f 0.85) was identified tentatively as ethylmalonic acid (R_f 0.85). The hydroxamate of the radioactive reaction product was prepared after conducting the enzymatic carboxylation reaction as described above. At the end of the incubation 1000 μmoles of neutralized hydroxylamine was added and after a 30-minute period of 0° the hydroxamates were extracted[10] and paper chromatographed as described above. A single radioactive spot (R_f 0.53) which also gave a purple color when sprayed with ferric chloride reagent was observed. Authentic ethylmalonyl monohydroxamate exhibited an R_f value (0.53) identical to that of the hydroxamate of the labeled reaction product. These results indicate that ethylmalonyl CoA is the product of the enzymatic carboxylation of butyryl CoA.

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RECEIVED OCTOBER 21, 1959

BIOLOGICAL CHLORINATION. IV. PEROXIDATIVE NATURE OF ENZYMATIC CHLORINATION[1]

Sir:

We have reported the extraction of a soluble β-ketoadipate chlorinase system from mycelial powders of *Caldariomyces fumago* which catalyzes the formation of δ-chlorolevulinic acid from β-ketoadipic acid and chloride ion.[2-4] Purification of these crude extracts revealed a multiple enzyme requirement and a heat stable cofactor requirement for enzymatic chlorination. As shown in Table I, after calcium phosphate gel treatment of the crude extract, a heat stable supernatant fraction and a heat labile gel eluate fraction are required for the formation of δ-chlorolevulinate.

(1) This work was supported in part by a grant (No. G-6463) from the National Science Foundation.

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TABLE I

HEAT STABLE FACTOR REQUIREMENT FOR β-KETOADIPATE CHLORINASE SYSTEM

The complete system contained 200 μmoles of potassium phosphate buffer, pH 4.8, 10 μmoles of KCl^{36} at a specific activity of 9,700 c.p.m. per μmole, 20 μmoles of potassium β-ketoadipate plus the additions listed below in a total volume of 1 ml. After 1 hour of incubation at 30° under aerobic conditions the reaction was stopped by the addition of 0.2 ml. of 7 N sulfuric acid. The formation of δ-chlorolevulinic acid was determined as described previously.[4]

	Additions	δ-Chlorolevulinate synthesis, mμmoles
1	0.9 mg. calcium phosphate gel eluate	7
2	4.5 mg. calcium phosphate gel supernatant	11
3	1 + 2	52
4	1 + heated supernatant	31
5	2 + heated eluate	6
6	1 + acid hydrolyzed supernatant	690
7	1 + 100 μmoles glucose	2,834
8	1 + 7 μmoles H_2O_2	1,200

Mild acid hydrolysis (0.1 N H_2SO_4, 1 hour, 120°) markedly enhances the activity of the heat stable fraction and active extracts contain large amounts of a polysaccharide[4] (glucose polymer). The hypothesis that the polysaccharide is the active factor is supported by the observation that glucose will replace the requirement for the heat stable fraction. Hydrogen peroxide also is active in replacing the heat stable fraction, indicative that the polysaccharide and its hydrolytic product, glucose, function in a hydrogen peroxide generating system. This has been established further by separation of the heat labile gel–eluate fraction into two enzymatic components on diethylaminoethyl cellulose columns. As shown in Table II both a glucose oxidase fraction plus a chloroperoxidase fraction are required for δ-chlorolevulinic acid formation from β-ketoadipic acid, chloride ion and glucose.

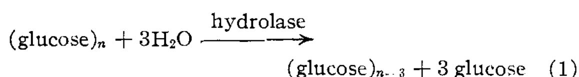
TABLE II

REQUIREMENT FOR HYDROGEN PEROXIDE GENERATING SYSTEM

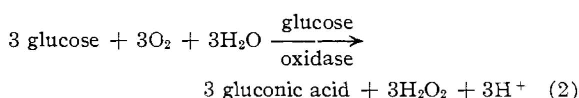
The conditions are the same as those described in Table I.

	Additions	δ-Chlorolevulinic acid synthesis mμmoles
1	Complete (2 through 6)	251
2	Complete minus 7 γ chloroperoxidase	13
3	Complete minus 14 γ glucose oxidase	28
4	Complete minus 100 μmoles glucose	4
5	Complete minus 20 μmoles β-ketoadipate	14
6	Complete minus 10 μmoles KCl	0

The enzymatic reactions leading to the formation of δ-chlorolevulinic acid in the crude extracts which contain catalase may therefore be formulated:



$$(\text{glucose})_n + 3H_2O \xrightarrow{\text{hydrolase}} (\text{glucose})_{n-3} + 3\ \text{glucose} \quad (1)$$



$$3\ \text{glucose} + 3O_2 + 3H_2O \xrightarrow[\text{oxidase}]{\text{glucose}} 3\ \text{gluconic acid} + 3H_2O_2 + 3H^+ \quad (2)$$