CVIII. CHOLEIC ACIDS.

IV. KETO-ENOL TAUTOMERISM AND CO-ORDINATION COMPOUNDS.

BY HARRY SOBOTKA AND JOS KAHN¹.

From the Laboratories of the Mount Sinai Hospital, New York.

(Received April 30th, 1932.)

AN important isomerism occurring in substances of biochemical interest is the keto-enol tautomerism. The peculiarities of the configurations

the state of equilibrium between both forms under varying conditions, and the rates of their interconversion have been elucidated by Meyer and co-workers [1911–20] in the case of ethyl acetoacetate and of some other substances of similar configuration. While Meyer and others have recognised and studied the position of the equilibrium in relation to the substituents R_1 and R_2 , and also the influence of temperature, solvent and acidity on the keto-enol equilibrium [Meyer and Kappelmeier, 1911; Meyer, 1912], nothing is known in regard to keto-enol equilibrium in co-ordination compounds.

Substances containing reactive methylene groups are endowed with unsaturated partial valencies and are, therefore, apt to enter into co-ordination compounds. Werner [1901] was the first to describe complex compounds of acetylacetone like

Many related compounds with various metallic salts have since been prepared, *e.g.* $\text{CH}_3\text{COCH}_2\text{COCH}_3 \cdot \text{AlBr}_3$ by Kohler [1902], $\text{C}_6\text{H}_5\text{COCH}_2\text{COCH}_3 \cdot \text{SbCl}_5$ from benzoylacetone, $\text{CH}_3\text{COCH}_2\text{COOC}_2\text{H}_5 \cdot \text{HSbCl}_6$ and $\text{CH}_3\text{COCH}_2\text{COOC}_2\text{H}_5 \cdot \text{SbCl}_5 \cdot 2\text{HSbCl}_6$ from ethyl acetoacetate by Rosenheim *et al.* [1903], the last containing 3 metal atoms for 1 molecule ethyl acetoacetate [*cf.* Pfeiffer, 1927]. The tautomeric substance has to be formulated as enol in the majority of these mixed inorganic-organic molecular compounds, and the enol hydrogen is replaced by one main valence of the metal. For other compounds, no decision regarding tautomerism was sought and we might attribute either configuration to purely organic co-ordination compounds like the compounds of ethyl acetoacetate with acid potassium trichloroacetate and with benzidine, $\text{CH}_3\text{COCH}_2\text{COOC}_2\text{H}_5$.

¹ Isidore Hershheim Research Fellow.

$(\text{CCl}_3\text{COO})_2\text{KH}$ [Koloseff, 1903] and $\text{CH}_3\text{COCH}_2\text{COOC}_2\text{H}_5 \cdot 2\text{NH}_2 \cdot \text{C}_6\text{H}_4 \cdot \text{C}_6\text{H}_4 \cdot \text{NH}_2$ [Heidrich, 1898].

While it is hardly possible to apply the bromine titration method of Meyer to these compounds, the co-ordination compounds of tautomeric substances with bile acids offer an opportunity for such a study. With this object in view, we prepared the choleic acids of ethyl acetoacetate, acetylacetone and benzoylacetone. These compounds are easily obtained by dissolving both the tautomeric substance and the desoxycholic acid in absolute alcohol.

The analysis of the diketone-choleic acids offered some difficulties, as the enol forms behave like weak acids and thus interfere with the estimation of the acid equivalents of the choleic acids. The alcoholic solutions of these diketones react acid to varying degrees, but their hydrogen ion dissociation and its relation to tautomerism has never been studied in a quantitative manner. Since no sharp end-point could be ascertained with bromothymol blue, a more acid indicator was employed in the determination of their acid equivalent. As desoxycholic acid on the other hand is not a very strong acid there was a limit on the acid side for the choice of the indicator. The most satisfactory end-point was obtained with bromocresol purple and this was used for benzoylacetone-choleic acid.

No consistent figures could be obtained for acetylacetone-choleic acid, because the enolised diketone will neutralise considerable amounts of alkali before the bile acid is completely neutralised. Neither could this choleic acid be titrated like the choleic acid of a fairly strong acid, as the enolisation of the keto-portion does not take place instantaneously. On the other hand, the back-titration with acid after having added an excess of alkali is not feasible, as there is no sharp demarcation between the dissociation of the first and second enol groups of this diketone.

The acid equivalents suggest a co-ordination number 3 for ethyl acetoacetate and for benzoylacetone. In order to corroborate this result we resorted to other analytical procedures. Ethyl acetoacetate on treatment with sulphuric acid decomposes to acetone and carbon dioxide. The acetone formed according to this reaction from ethyl acetoacetate-choleic acid was determined by the method of Engfeldt [1917]. This analysis conformed with the assumption of a tricholeic acid. Another sample of ethyl acetoacetate-choleic acid was subjected to repeated steam-distillations under reduced pressure until the non-volatile residue attained constant weight. The loss of volatile matter tallied with the percentage of ethyl acetoacetate calculated for a tricholeic acid.

The enol titration of these choleic acids yielded striking results. When they were dissolved rapidly in cold alcohol and treated with alcoholic bromine solution, the following enol percentages were obtained: 8.4 % for ethyl acetoacetate-choleic acid, 11.95 % for benzoylacetone-choleic acid and 7.75–7.9 % for acetylacetone-choleic acid. The computed percentages of acholic component for tricholeic acids are in the order given, 9.95 %, 12.1 % and 7.85 %. A comparison of these two sets of figures permits the following conclusions. (1) These compounds, including the one of acetylacetone for which no other analytical evidence was accessible, contain three molecules desoxycholic acid for one molecule of the tautomeric substance, (2) The tautomeric constituent is bound in completely enolised state.

In alcoholic solution more than 99 % of benzoylacetone is present in the enol form. Thus, the complete enolisation of this substance in molecular

combination with a bile acid is not surprising and may serve as a control. Acetylacetone in the liquid state is a mixture of 80 % enol and 20 % ketone, while the enol form is increased to 84 % after a solution in absolute alcohol has attained equilibrium. We found 76 % enol after prolonged standing in 95 % alcohol. The figures for freshly dissolved acetylacetone-choleic acid are distinctly higher; they range between 91 and 101 %, and support the assumption that acetylacetone is completely enolised in its co-ordination compound.

Ethyl acetoacetate contains 7.4 % enol in undiluted form, 12 % in absolute alcohol, 8.7 % in 96 % alcohol and 8.0 % in 95 % alcohol. In sharp contrast to these data, ethyl acetoacetate-choleic acid contained as much as 84.6 % enol at the time when bromine was added. This figure and its rapid decrease within a few minutes implies that this choleic acid is likewise completely enolised, although its original enol share of 100 % changes so rapidly as to escape experimental verification. It is noteworthy in this connection that the benzoylacetone-choleic acid is completely precipitated to the point of solidification immediately upon mixture of concentrated alcoholic solutions of its constituents. The acetylacetone-choleic acid is formed at a slower rate, but after 5–10 minutes the concentrated alcoholic solution likewise solidifies. The ethyl acetoacetate-choleic acid crystallises overnight. Thus, the rate of formation of these choleic acids increases with the enol content of the free tautomeric component.

If the solutions of these choleic acids are allowed to stand for prolonged intervals before determination of the enol, considerable ketonisation takes place in the case of acetylacetone and ethyl acetoacetate. The final enol percentage is about 13 % for the ester-choleic acid in 95 % alcohol, and 15 % in absolute alcohol, while the enol content of acetylacetone-choleic acid drops to about 70 %. These figures differ slightly from the keto-enol equilibrium of the free substances, but they represent true equilibria, as an equal enol percentage is reached in a solution resulting from combination of alcoholic solution of ethyl acetoacetate and of desoxycholic acid.

These observations may be interpreted in regard to the molecular dissociation of choleic acids in solution. Unpublished experiments on the molecular weight of choleic acids of simple aliphatic acids favour the assumption that choleic acids, when dissolved in alcohol, completely dissociate into their molecular components. Thus, we are disinclined to attribute the difference in the final equilibrium between solutions of free and of combined ethyl acetoacetate to the existence of choleic acid in solution. Molecular dissociation of these choleic acids is practically instantaneous. Repeated experiments demonstrated beyond doubt that very rapid manipulation leads to lower enol figures than the maximal figures given above. This observation applies to all three substances used. When less than 30 seconds elapsed between solution of the choleic acid and the addition of the bromine solution, enol values of 73.8, 91 and 75 % were obtained for ethyl acetoacetate-, acetylacetone-, and benzoylacetone-choleic acid respectively. The deviation from 100 % appears

to be due to the presence of undissociated choleic acid, in which one can imagine the double bond of the enol group screened from the bromine by the surrounding bile acid molecules. Studies on the kinetics of tautomerisation of these substances are reported elsewhere [Sobotka and Kahn, 1932].

Another case of tautomerism which we studied in this connection was that of anthranol (9-hydroxyanthracene) and anthrone (9-ketodihydroanthracene) [Meyer, 1910]. These substances do not tautomerise as readily as ethyl acetoacetate and the aliphatic diketones. They have been isolated separately. The ketone is a white substance, while the enol is deep yellow and displays a brilliant blue fluorescence in solution. The disappearance of this fluorescence serves as indicator in the titration of anthranol-anthrone mixtures with dilute alcoholic bromine solution [Meyer and Sander, 1912-13].

We synthesised a choleic acid from an alcoholic solution of anthrone which had reached equilibrium at about 15 % enol. The crystals obtained were almost white and showed no fluorescence initially when dissolved in alcohol. After $1\frac{1}{2}$ hours, the solution had developed a strong blue fluorescence and the enol content was 14 % according to a bromine titration. This enolisation could be hastened by addition of a trace of hydrochloric acid and by heating. It follows from these observations, that in this instance the choleic acid contains the keto-form of the desmotropic pair.

Tautomerism is one instance in which the presence of a substance in two forms of varying reactivity can be demonstrated by analysis and preparative isolation. But there are many other substances in which the rearrangement into a more reactive form is conjectured without direct proof. It is a function of catalysts to elicit or aid such rearrangements. The present experiments exemplify how a substance may be converted into a very reactive form by the rather gentle reaction of molecular combination with bile acid. This might serve as an example of one of the many functions of bile acids *in vivo*. The demonstration of the enolising influence exercised by bile acids on free acetoacetic acid and other β -keto-acids offers experimental difficulties. It is plausible however to attribute to the bile acids a function in the intrahepatic β -oxidation of fatty acids through enolisation of the intermediary keto-acids.

EXPERIMENTAL PART.

Benzoylacetone-choleic acid was prepared by mixing a solution of 1.5 g. of benzoylacetone of m.p. 61° in 10 cc. absolute alcohol with a solution of 10 g. desoxycholic acid in 25 cc. alcohol. The choleic acid precipitated and the solution solidified. The substance was recrystallised from alcohol and melted at 185° (corr.). 88.4 mg. and 92.4 mg. dissolved in alcohol titrated in the presence of bromocresol purple required 3.96 cc. and 4.36 cc. 0.05 *N* NaOH. The acid equivalent is therefore 446-447, while the acid equivalents calculated for di-, tri- and tetra-choleic acids are 473, 446 and 433 respectively. (The bromine titrations are reported under a separate heading.)

Ethyl acetoacetate-choleic acid was prepared in an analogous manner. The crystals were colourless and melted at 154° (corr.). 203.5 mg. in ethyl alcohol required 4.58 cc. 0.05 *N* NaOH with bromocresol purple, and 4.68 cc. with bromothymol blue. The acid equivalents calculated from these figures are 444.5 and 435. The theoretical value for tricholeic acid is 435.6. In addition to this analysis, 256.4 mg. ethyl acetoacetate-choleic acid were digested with 10 cc. 20 % sulphuric acid for 10 hours on the water-bath. The solution was then treated according to the method of Engfeldt [1917]. 10.75 cc. 0.1 *N* iodine used corresponded to 10.4 mg. acetone or 23.1 mg. ethyl acetoacetate, while the calculated values were 11.3 mg. acetone or 25.4 mg. ester.

The desoxycholic acid contained in this choleic acid was determined as follows. 518.8 mg. choleic acid were placed with 10 cc. water in a small double-neck distilling flask which had been weighed with a capillary tube and rubber stopper. The water was distilled off under reduced pressure, it was then replaced three times and each time removed to dryness until the distillate failed to give the purple reaction with FeCl₃ characteristic for ethyl acetoacetate. The flask had lost 52.0 mg. or 10.02 % of the weighed amount of choleic acid. Its theoretical percentage of ethyl acetoacetate is 9.95 %.

Acetylacetone-choleic acid was prepared by adding 1.5 g. of the diketone to 11 g. desoxycholic acid in 30 cc. absolute alcohol. The yield was 5.7 g. of crystals melting at 162°.

Enol titration. The titration of the enol was carried out by dissolving a weighed amount of substance in chilled alcohol. After the time given in Table I had elapsed, 15–25 cc. of an approximately 0.2 *N* alcoholic solution of bromine was added rapidly. The reaction of the bromine with the unsaturated compound takes place at once and the excess bromine is removed by addition of 2 cc. of a 10 % alcoholic solution of β-naphthol which instantly decolorises the mixture. 1 cc. of glacial acetic acid and about 5 cc. of an aqueous 20 % solution of potassium iodide cause the liberation of free iodine and the completion of the reaction is insured by placing the Erlenmeyer flask for 10 minutes on the water-bath. The iodine is then titrated with 0.1 *N* thiosulphate without indicator. Another 10 minutes on the water-bath will bring out an additional 0.1 to 0.3 cc. 0.1 *N* iodine. The amount of enol equivalent to 1 cc. 0.1 *N* thiosulphate, is calculated from

$$\frac{\text{CH}_3.\text{C}(\text{OH})\text{:CH}.\text{COOC}_2\text{H}_5}{2 \times 10}$$

or 65 mg., for acetylacetone 50 mg., and for benzoylacetone 81 mg.

The results of these titrations are summarised in Table I. All titrations were carried out in 95 % alcohol.

Anthrone-choleic acid was prepared by dissolving 3 g. desoxycholic acid in a solution of 0.5 g. anthrone in 10 cc. alcohol. Upon standing, pale crystals of m.p. 179° separated. Their acid equivalent was 441. The theoretical acid equivalent for an anthrone-tetracholeic acid is 437.8.

Table I. *Enol titration of choleic acids with a tautomeric component.*

Substance	Time elapsed between solution and addition of of bromine	Amount of substance (mg.)	0.1 <i>N</i> thio- sulphate (cc.)	Enol form		
				(mg.)	% of	
					choleic acid	tautomeric component
Benzoylacetone- choleic acid (0°)	10 secs.	505	5.70	46.2	9.1	75
	10 secs.	491	6.00	48.6	9.9	82
	1 min.	621	9.15	74.2	11.95	99
Acetylacetone- choleic acid (0°)	10 secs.	453	6.25	31.3	7.25	91
	1 min.	403	6.23	31.2	7.75	99
	2 mins.	408	6.45	32.3	7.9	100
	1 hr.	298	4.32	21.6	7.25	91
	48 hrs.	447	4.75	23.7	5.3	69
Ethyl acetoacetate- choleic acid (0°)	10 secs.	358.5	5.43	26.3	7.34	73.8
	20 secs.	563	6.65	43.2	7.68	77.2
	30 secs.	299	3.87	25.2	8.43	84.7
	30 secs.	298	3.72	24.2	8.12	81.6
	2 mins.	472	5.06	32.9	6.97	70.0
	4 mins.	472	4.13	26.8	5.68	57.1
	11 mins.	536	1.97	12.8	2.39	24.0
	48 hrs.	472	1.00	6.5	1.38	13.8
Ethyl acetoacetate- choleic acid (–10°)	2 mins.	308.5	3.80	24.7	8.01	80.5
	30 mins.	308.5	3.30	21.4	6.94	69.7
	75 mins.	308.5	2.90	18.9	6.13	61.6
	17 hrs.	308.5	0.65	4.2	1.36	13.6
Ethyl acetoacetate <i>plus</i> desoxycholic acid (0°)	17 hrs.	{ 500 5000 }	10.45	68.0	—	13.6
Desoxycholic acid (0°)		500	<0.10	—	—	—

SUMMARY.

The preparation and analysis of co-ordination compounds consisting of three molecules desoxycholic acid and one molecule of a tautomeric substance are described. While these substances in the free state contain varying proportions of keto-form, they are completely enolised when bound in choleic acids. However, anthrone-tetracholeic acid contains the acholic component in the keto-form.

There is evidence of rapid and complete molecular dissociation when these choleic acids are dissolved. The bearing of these observations on the mechanism of β -oxidation is discussed.

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Addendum (June 29th, 1932). Our conceptions dovetail perfectly with a hypothesis which Fowweather reached from quite a different source [*Biochem. J.* 1932, **26**, 165]. He suggests that tautomerism may account for the difference between bilirubin preparations giving a direct van den Berg reaction and those giving only an indirect one. Fowweather adduces evidence that promptly diazotisable bilirubin, as obtained from bile, is the enol form and he concludes that some bile constituent, presumably bile acid, enolises the pigment and has a stabilising influence on its enol form.