Short Communications

Modification of Emulsin Action on Asparaginy-Carbohydrate from Ovalbumin by Dinitrophenylation

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Information about the structures of biopolymers is frequently derived from an examination of the products of graded hydrolysis with either chemical or enzymic catalysts. The degraded molecules are less complicated and more amenable to constitutional studies. Whereas acid catalysis results in a somewhat random cleavage, the enzymes, even such crude mixtures as the emulsins, show a more specific hydrolysis. An example is described below in which the action of sweet-almond emulsin on amino acyl-carbohydrate is modified by the dinitrophenylation of the amino acid residue.

Experimental. The asparaginy-carbohydrate of ovalbumin was isolated by digestion with Pronase at 40–50° for 40–48 hr. in 0.05M-phosphate or 0.1M-tris buffers at pH 7.4–7.8, 1 mM with respect to Ca^{2+} ions. The glycopeptides were isolated quantitatively by gel-filtration and fractionated by zone electrophoresis for 21 hr. at 800 v in *N*-acetic acid (Lee & Montgomery, 1962). After electrophoresis, the glycopeptides were eluted with *N*-acetic acid as two completely separated components, asparaginy(-carbohydrate)leucine [Asp(NH·Carb)-Leu] at effluent volume 55–105 ml. (peak effluent volume 70 ml.) and asparaginy-carbohydrate [Asp(NH·Carb)] at effluent volume 110–160 ml. (peak effluent volume 130 ml.). Further purification of Asp(NH·Carb) was achieved by chromatography on DEAE-Sephadex, eluting with CO_2 -saturated water. Asp(NH·Carb) was composed of the anhydro residues of D-mannose, 51.4%, *N*-acetyl-D-glucosamine (determined as glucosamine), 38.6%, and asparagine (determined as aspartic acid), 7.9%. It showed a molecular weight of 1560, obtained by using a thermoelectric osmometer (Brady, Huff & McBain, 1951) (model 301A from Mechrolab, Mountain View, Calif., U.S.A.).

D-Mannose was determined by both the phenol-sulphuric acid (Dubois, Gilles, Hamilton, Rebers & Smith, 1956) and the resorcinol-sulphuric acid (Devor, Conger & Gill, 1958) methods, which agreed within 5% of each other. D-Glucosamine and the amino acid residues were analysed by the method of Moore, Spackman & Stein (1958). The composition of the oligosaccharide fragments was also checked after acid hydrolysis (in 0.03N-HCl for 25 hr.

at 110°) by quantitative paper chromatography (in ethyl acetate-acetic acid-formic acid-water (18:3:1:4, by vol.), analysing for D-mannose and D-glucosamine by the method of Park & Johnson (1949). The latter submicro procedure, although not as accurate as those given previously, permitted the molar ratios of the sugar residues in oligosaccharides to be determined within 10–15%; the molar ratio of D-mannose to D-glucosamine in Asp(NH·Carb) and Asp(NH·Carb)-Leu was 5.0:2.6 and 5.0:2.7, respectively, when analysing less than 1 mg. amounts.

Dinitrophenylation of Asp(NH·Carb) in 0.1M-NaCl with 1-fluoro-2,4-dinitrobenzene was followed at 40° and pH 8.0 by a Radiometer TTT 1a pH-stat, by using 0.1N-KOH delivered from a micro-syringe. The final reaction mixture was desalted by gel-filtration. DNP-Asp(NH·Carb) showed a molar extinction coefficient of 15.0×10^3 at 340 m μ in acetic acid, 15.1×10^3 at 335 m μ and 15.4×10^3 at 360 m μ in water, and 15.0×10^3 at 355 m μ in 0.1N-ammonia.

Digestion of Asp(NH·Carb) with sweet-almond emulsin (Worthington Biochemical Corp.) was carried out in 0.01M-sodium acetate buffer, pH 4.1 at 38–40°. Blanks of glycopeptide and of emulsin were run concomitantly. Periodically, samples of each solution were passed through Dowex 50 (X2; H^+ form) to remove any residues still linked to the amino acid. The effluent was analysed for *N*-acetyl-D-glucosamine (Morgan & Elson, 1934) and total D-mannose and hexosamine. After 9–12 days the assay for total D-mannose showed that most of the sugar was in the effluent and the remaining emulsin digest was fractionated on a charcoal-Celite column by a stepwise elution with aqueous ethanol up to 15% (v/v) concentration. The fraction eluted with 15% ethanol was then separated further by paper chromatography in the usual way, developing with ethyl acetate-pyridine-water (12:5:4, by vol.). The individual components were eluted with water and the component sugars were determined before and after reduction with NaBH_4 . The digestion of DNP-Asp(NH·Carb) with emulsin was conducted similarly, the whole digestion mixture being separated directly by paper chromatography in butan-1-

ol-acetic acid-water (4:1:1, by vol.) for 25 hr. Some colourless fragments were detected on a guide strip with AgNO_3 reagent.

Results and discussion. The asparaginy-carbohydrate fragment from ovalbumin is shown in the present study and by other workers (Johansen, Marshall & Neuberger, 1961; Kaverzneva & Tsi, 1961; Nuenke & Cunningham, 1961) to be composed of 5 mol.prop. of D-mannose, 3 mol.prop. of N-acetyl-D-glucosamine and 1 mol.prop. of asparagine. The linkage with asparagine involves the reducing group of one of the N-acetyl-D-glucosamine residues as a β -aspartyl-glycosylamide, the same as is probably found in ovomucoid (Montgomery & Wu, 1963; Tanaka, 1961), γ -globulin (Rosevear & Smith, 1961) and orosomucoid (Kamiyama & Schmid, 1962). It serves therefore as a model to study the action of emulsin.

The digestion of Asp(NH·Carb) with sweet-almond emulsin results in the gradual cleavage of the β -aspartyl bond until after 9–12 days all the carbohydrate fragments have been hydrolysed from the aspartic acid residue. Direct analysis of the emulsin digest showed the liberation of 90 % of the theoretical amount of free aspartic acid. Under the same conditions the DNP-Asp(NH·Carb) gave no evidence of free aspartic acid, asparagine or their DNP derivatives, and the components in the hydrolysate did not change qualitatively during 9–22 days, clearly demonstrating the resistance of the asparaginy bond when the α -amino group of the amino acid is blocked by dinitrophenylation. It has not yet been determined whether other blocking groups modify the action of emulsin similarly.

The preliminary examination of the products of the emulsin action on these two substrates also reflects a difference. Charcoal-column chromatography of the Asp(NH·Carb) digest showed that mannose was eluted with water and that the other principal fraction was eluted with 15 % ethanol. The composition of the components of the latter fraction, when separated further by paper chromatography, corresponded to $(\text{Man}_2, \text{GNac}) \rightarrow \text{GNac}$ (1 %), $(\text{Man}_3, \text{GNac}) \rightarrow \text{GNac}$ (2 %), $(\text{Man}_4, \text{GNac}) \rightarrow \text{GNac}$ (17 %), and $(\text{GNac}, \text{Man}_4, \text{GNac}) \rightarrow \text{GNac}$ (12 %), where GNac represents N-acetylglucosamine and the recoveries of mannose are given in parentheses. The digest of DNP-Asp(NH·Carb) contained approx. 85 % of unchanged substrate, together with six components containing a DNP residue and four without it. Of these the principal products were $\text{GNac} \rightarrow \text{NH-Asp(DNP)}$ (6 %) and $(\text{Man}_5, \text{GNac}) \rightarrow \text{GNac} \rightarrow \text{NH-Asp(DNP)}$ (4 %), with another product that contained no mannose and is probably $\text{GNac} \rightarrow \text{GNac} \rightarrow \text{NH-Asp(DNP)}$. The above results can be compared with the action of 0.1 N-HCl for 165 min. at 100° on DNP-Asp(NH·Carb). The principal product was DNP-

Asp. Some of the other 13 DNP-labelled fragments were $\text{GNac} \rightarrow \text{NH-Asp(DNP)}$ (13 %), $\text{GNac} \rightarrow \text{GNac} \rightarrow \text{NH-Asp(DNP)}$ (2 %), $\text{Man} \rightarrow \text{GNac} \rightarrow \text{NH-Asp(DNP)}$ (0.7 %), and $\text{Man} \rightarrow \text{Man} \rightarrow \text{GNac} \rightarrow \text{NH-Asp(DNP)}$ (0.3 %), the recoveries being calculated from the extinction due to the DNP group.

By modifying one proposed structure for the carbohydrate of ovalbumin (Clamp & Hough, 1963) in the light of the above information to the monosaccharide sequence $\text{GNac} \rightarrow \text{Man} \rightarrow \text{GNac} \rightarrow \text{GNac} \rightarrow (\text{Man}, \text{Man}(\text{Man}), \text{Man})\text{NH-Asp}$, it is seen that all the reported fragments from Asp(NH·Carb), once freed from the asparagine, can be the result of mannosidase activity, whereas those from the DNP derivative involve also an N-acetyl-D-glucosaminidase.

The modification of the action pattern of emulsin, together with partial acid hydrolysis, will enable a wide spectrum of oligosaccharide-cleavage fragments to be obtained from glycopeptides, with resulting advantages in carbohydrate structural studies. Further, for such studies it is advantageous to be able to locate the origin of the cleavage fragments in the original glycopeptide by having the 'reducing end' of the oligosaccharides identified with aspartic acid, or its DNP derivative, which also permit the ready expression of the molar ratios of the sugar residues.

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The Determination of Methyl Esters of Polyunsaturated Acids by Gas-Liquid Chromatography

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Published data on the fatty acid composition of lipids by using gas-liquid chromatography indicate in some instances the presence of considerable amounts of polyunsaturated acids without adequate confirmation by other means. A survey of the fatty acid composition of New Zealand tallows revealed that the iodine values calculated from the composition of the methyl esters as determined by gas-liquid chromatography were up to 10% lower than those determined by the Wijs procedure. This loss could be related to a loss of methyl linolenate. Such a possibility is indicated by gas-liquid-chromatographic results of Craig & Murty (1959). Using 60-80 mesh Celite coated with either succinate or adipate diethylene glycol polyester and a thermal conductivity detector, they found only 9.4-9.8% of linolenic acid in a synthetic mixture containing 11% of this constituent. Their results, however, showed a relatively smaller loss with a larger amount of linolenic acid. The relatively larger losses of methyl linolenate shown in Table 1 are associated with the smaller samples (0.1 μ l.) used in the present work as compared with 1-3 μ l. used by Craig & Murty (1959).

The low values obtained for methyl linolenate made it desirable to examine the values for methyl esters of other polyunsaturated acids. For this purpose shark-liver oil, which is known to be rich in C₂₀ and C₂₂ polyunsaturated acids, was selected. It was found that whereas the methyl esters of the hydrogenated oil showed 14.5% of *n*-C₂₀ acids and 25.6% of *n*-C₂₂ acids, the values for the unhydro-

Table 1. *Loss of methyl linolenate in methyl linolenate-methyl stearate mixtures*

The analyses were carried out with 20% diethylene glycol adipate polyester on 30-80-mesh Celite 545 at 185° with an Argon ionization detector and a flow rate of 30-35 ml./min. The losses of methyl linolenate are calculated on the basis of the peak areas found compared with those calculated from the known composition.

Sample	Peak area		Loss (%)
	Calculated	Found	
1	76	35	53.9
2	79	40	49.4
3	116	52	55.2
4	120	60	50.0
5	176	90	48.9
6	226	111	50.9
		Mean	51.4

genated oil were 7.4 and 3.6% respectively. On this basis the loss of C₂₀ acids amounted to 51%, whereas that of the C₂₂ acids was 86%. These results emphasize the desirability of confirming by methods other than gas-liquid chromatography the amounts of the polyunsaturated constituents.

Investigations in progress indicate that the loss of polyunsaturated acids may be largely eliminated by the use of less-polar solid supports without recourse to coating with surface deactivators such as hexamethyldisilazane.

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Effect of Puromycin and Insulin on Amino Acid Accumulation by Isolated Intact Rat Diaphragm

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In circumstances similar to that in which it increases the incorporation of labelled amino acids into protein of isolated rat diaphragm, insulin also enhances the accumulation of three ¹⁴C-labelled non-utilized amino acids (α -aminoisobutyric acid,

cycloleucine and isovaline) and of some (glycine, proline, hydroxyproline, serine, methionine, threonine), but not all, of the utilized amino acids (cf. Wool, 1964, for references). There is nothing of the structure, metabolism or transport in muscle of

these amino acids that distinguishes them from the others not influenced by insulin and allows systematic sense to be made of the findings.

The tacit assumption has always been made, in studying the accumulation of ^{14}C -labelled amino acids, that the amount of amino acid incorporated into protein is only a small fraction of that added to the medium and may, therefore, safely be ignored. Clearly, this assumption needs testing: for that purpose what is required is a substance that has an effect on muscle analogous to the action of propylthiouracil on the thyroid. (Propylthiouracil blocks the organic binding of iodide but does not interfere with its transport into the thyroid; it, thereby, allows study of the transport step in isolation from thyroxine synthesis.) Puromycin would seem to be eminently suited for the purpose; in proper concentrations the antibiotic will all but completely inhibit protein synthesis (Yarmolinski & de la Haba, 1959) but it does not interfere with the transport of α -aminoisobutyric acid in muscle (Fritz & Knobil, 1963). We have tested the effect of insulin on the accumulation of amino acids ordinarily not responsive to the hormone. In the presence of sufficient puromycin to suppress protein synthesis, the accumulation of the five amino acids tested are all now increased by insulin.

The results are in Table 1. In the presence of puromycin insulin significantly stimulates accumulation by isolated intact rat diaphragm of radioactivity from ^{14}C -histidine, -leucine, -phenylalanine, -tyrosine and -alanine; in the absence of

puromycin insulin has no such an effect (Manchester & Young, 1960; Akedo & Christensen, 1961), a finding verified here for ^{14}C -histidine. Puromycin does not itself alter the uptake of α -aminoisobutyric acid by rat diaphragm (Fritz & Knobil, 1963); we have found a small, but not significant, increase in the transport of the natural amino acids into muscle in the presence of puromycin alone (cf. the data for ^{14}C -histidine, Table 1). The amount of puromycin used ($200\text{ }\mu\text{g./ml.}$) inhibits amino acid incorporation by more than 99% and completely suppresses the insulin stimulation of protein synthesis, a finding that indicates that the influence of insulin on the two processes, amino acid transport and protein synthesis, need not of necessity be coupled—a point made before by Fritz & Knobil (1963).

The results provide at least a partial solution to the problem of why insulin increases accumulation of some utilized amino acids and not others. Apparently, in the presence of insulin the increase in the rate of incorporation into protein of most, but not all, amino acids is sufficient to prevent their accumulation to a concentration greater than that which occurs in the absence of the hormone, even when transport too is stimulated. Puromycin blocks amino acid incorporation into protein and thereby uncovers the insulin stimulation of amino acid transport. The antibiotic would seem then to be a valuable tool in the study of the mechanism and the regulation of amino acid transport just as propylthiouracil has proven of value in the study of

Table 1. *Effect of puromycin and insulin on accumulation and incorporation into protein of radioactivity from ^{14}C -labelled amino acids by isolated intact rat diaphragm*

Additions to the medium			Accumulation (counts/min./ml. of tissue water) (counts/min./ml. of medium)	Incorporation (counts/min./mg. of protein)
^{14}C -labelled amino acid	Puromycin	Insulin		
L-Histidine	—	—	4.75 ± 0.214	234 ± 16.9
	—	+	4.85 ± 0.116	$394 \pm 20.5^{**}$
	+	—	5.07 ± 0.297	$2 \pm 0.2^{\dagger}$
	+	+	$6.83 \pm 0.277^{*}$	2 ± 0.3
L-Leucine	+	—	1.41 ± 0.053	30 ± 2.5
	+	+	$1.71 \pm 0.034^{*}$	36 ± 5.9
	+	—	1.67 ± 0.020	.
L-Phenylalanine	+	+	$1.97 \pm 0.010^{**}$	.
	+	—	3.15 ± 0.070	.
L-Tyrosine	+	+	$3.74 \pm 0.094^{*}$	.
	+	—	1.89 ± 0.115	.
L-Alanine	+	+	$2.57 \pm 0.095^{*}$	.

* A difference due to insulin that is significant, $P < 0.01$.

** A difference due to insulin that is highly significant, $P < 0.001$.

$\dagger$ Inhibition by puromycin is 99.2%.

iodide transport and thyroxine synthesis in the thyroid.

The results do not, of course, prove that insulin increases protein synthesis in muscle solely by an influence on amino acid accumulation. There is, in fact, evidence that leads to the opposite conclusion, namely, that insulin can enhance protein synthesis in muscle by an action at a site distal to amino acid transport, presumably by an effect on some intracellular process (Wool & Krah1, 1959*b*, 1964; Manchester & Krah1, 1959).

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Biosynthesis of L-Tyrosine O-Sulphate by a Novel Route

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L-Tyrosine O-sulphate occurs as a unique component of several mammalian fibrinogens (e.g. Jevons, 1963). It is also present in normal human urine in unexpectedly large amounts (Tallan, Bella, Stein & Moore, 1955). Segal & Mologne (1959) have provided some indirect evidence that the carboxyl group of L-tyrosine must be blocked, as in the methyl and ethyl esters, before the amino acid can act as a sulphate acceptor in the liver adenosine 3'-phosphate 5'-sulphatophosphate-phenolsulphotransferase system, although in neither case was the nature of the sulphated product checked. We have now established that the product of the sulphation of either the methyl or ethyl ester of L-tyrosine by rat-liver supernatant preparations is free L-tyrosine O-sulphate.

Suspensions (20 %) of rat liver in 0.15M-KCl were prepared with the aid of a glass homogenizer and centrifuged at 100 000*g*_{av} for 90 min. The supernatant (designated 'whole supernatant') was adjusted to pH 7.2 and stored in 1 ml. quantities at -10°. In other experiments, rat livers were homogenized in 0.05M-KCl (7 ml./5 g. of liver) and the suspension was then diluted with a further 2 vol. of 0.05M-KCl before centrifuging at 100 000*g*_{av} for 50 min. The supernatant was diluted sixfold with 0.05M-KCl and the pH adjusted to 5.1 with N-

acetic acid. Precipitated protein was collected by centrifuging at 15 000*g*_{av} for 10 min., resuspended (glass homogenizer) in 0.1M-tris (1 ml./g. of original liver) that had been adjusted to pH 7.6 with a trace of HCl, and insoluble material removed by centrifuging. The supernatant ('pH 5 supernatant') was stored in 1 ml. quantities at -10°.

With both types of supernatant the following standard reaction mixture was used: supernatant preparation (100 μ l.), ATP (4 μ moles), MgCl₂ (2 μ moles), cysteine hydrochloride (1 μ mole), Na₂³⁵SO₄ (10 μ c) and acceptor (0.5 μ mole, dissolved in 10 μ l. of 0.5M-KH₂PO₄). All components were adjusted to pH 7.2 with 2N-NaOH and the final volume of the mixture was 160 μ l. After incubation for 1 hr. at 38° the reaction tubes were placed briefly in a boiling-water bath and precipitated protein was removed by centrifuging.

In the absence of added acceptor, both whole supernatant and pH 5 supernatant synthesized ³⁵S-labelled adenosine 3'-phosphate 5'-sulphatophosphate which was identified by radioautography after paper electrophoresis (0.1M-acetate buffer, pH 4.5; potential gradient 11 v/cm.) or paper chromatography with butan-1-ol-acetic acid-water (50:12:25, by vol.) or isobutyric acid-0.3N-NH₃ (5:3, v/v). Radioautograms also showed that

endogenous sulphate acceptors were present in the enzyme preparations (cf. Spencer, 1960).

Both enzyme preparations were able to use *p*-nitrophenol as sulphate acceptor but neither could use L-tyrosine for this purpose. However, incubation of the pH 5 supernatant with L-tyrosine methyl ester resulted in the appearance of two new radioactive spots on chromatograms and electrophoresis strips. One of the spots exhibited mobilities identical with those of chemically synthesized L-tyrosine $O[^{35}\text{S}]$ -sulphate, and the other spot was assumed to be the *O*-sulphate ester of L-tyrosine methyl ester. Two radioactive spots were also present on chromatograms when L-tyrosine ethyl ester was used as acceptor, one of them showing the same mobilities as L-tyrosine $O[^{35}\text{S}]$ -sulphate. The other was assumed to be the *O*-sulphate ester of L-tyrosine ethyl ester.

When whole supernatant was used as enzyme source with either the methyl or ethyl ester of L-tyrosine as acceptor, one new spot only (mobilities identical with L-tyrosine $O[^{35}\text{S}]$ -sulphate) appeared on chromatograms or electrophoresis strips.

Firm proof that L-tyrosine $O[^{35}\text{S}]$ -sulphate was the sole end product of the biosynthetic process in whole-supernatant preparations was obtained as follows. The quantities of the components of the standard reaction mixture were increased fivefold and L-tyrosine methyl ester was used as acceptor. After incubation and removal of protein as described above, the mixture was applied to a column (13 cm. $\times$ 2.5 cm.) of Dowex 50 resin (200–400 mesh; H^+ form). The column was washed with water and the eluate collected in 1 ml. fractions. Two peaks of radioactivity were obtained, the first (mainly inorganic $[^{35}\text{S}]$ sulphate) in the initial 4 ml. of eluate, the other between 6 and 8 ml. The first peak only was obtained in control experiments in which L-tyrosine methyl ester was omitted from the reaction mixture. The second-peak eluate was neutralized with ammonia, freeze-dried and a small sample submitted to paper electrophoresis. A single radioactive component was present which moved at the same rate as authentic L-tyrosine $O[^{35}\text{S}]$ -sulphate. The remainder of the freeze-dried material was then mixed with 90 mg. of unlabelled L-tyrosine *O*-sulphate and the whole was recrystallized six times. After each recrystallization a sample was removed for determination of ^{35}S as described by Dodgson, Powell, Rose & Tudball (1961). Material of constant specific radioactivity was obtained after the first recrystallization. The final product was examined by paper electrophoresis, giving one ultraviolet-absorbing spot which was also radioactive and which moved as expected for L-tyrosine *O*-sulphate.

Considerable difficulty has been experienced in obtaining unequivocal proof that the sulphate ester of L-tyrosine methyl ester is the second radioactive component which is formed when L-tyrosine methyl ester is used as acceptor in the pH 5-supernatant system. Thus it has proved impossible to prepare the sulphate ester by chemical synthesis although a great many different approaches to the problem have been made. Space does not permit discussion of the formidable technical difficulties involved.

Strong support for the assumed identity of the metabolite was obtained by eluting the metabolite from chromatograms and incubating the eluate with whole supernatant in the presence of 0.1M-tris-acetate buffer, pH 7.2, for 1 hr. at 38°. Paper chromatography and electrophoresis of the test solutions showed that the metabolite disappeared as a result of this treatment, and free L-tyrosine $O[^{35}\text{S}]$ -sulphate was formed. No such changes were detected in control experiments without enzyme.

The collective results lead to the conclusion that the methyl (or ethyl) ester of L-tyrosine can act as an acceptor for sulphate in the adenosine 3'-phosphate 5'-sulphatophosphate-phenolsulphotransferase system. However, the initial product then loses the methyl (or ethyl) group as the result of the action of an enzyme which is present in the whole supernatant but which is present in limited amounts only in the pH 5 supernatant. Whether this enzyme is a simple esterase or a proteolytic enzyme with esterase activity (cf. Myers, 1960) is not yet known. If the latter proves to be the case it might point to the possibility that simple di- or tripeptides containing an L-tyrosine residue in the *N*-terminal position may undergo biological sulphation *in vivo*, followed by proteolysis to yield free L-tyrosine *O*-sulphate. Such a possibility might help to account for the high concentrations of the ester that are present in normal human urine and that cannot possibly be explained by the daily turnover of fibrinogen.

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Interactions between Histones and Nucleic Acids

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Histones inhibit the biosynthesis of RNA formed on a DNA template in the presence of RNA polymerase (Huang & Bonner, 1962, 1963; Barr & Butler, 1963; Hindley, 1963). A number of well-characterized histone fractions, termed f1 (lysine-rich), f2 (a) and f2 (b) (intermediate), and f3 (arginine-rich), have been isolated (Johns, Phillips, Simson & Butler, 1960; Johns & Butler, 1962), and it has been suggested that they may distinguish different classes of genes (Butler, 1963). It was found, however (Barr & Butler, 1963), that the inhibition of RNA synthesis by these fractions was comparatively non-specific since f1 in sufficient amount caused a total inhibition of RNA synthesis, and the other fractions, though less effective, caused more inhibition than would be expected if they acted only on that part of the DNA with which they are combined in the native nucleoprotein.

To examine the nature of the specific interaction between these histone fractions and parts of the DNA, a study has been made of the precipitation of DNA by the different histone fractions.

To solutions of DNA (0.5 mg. in 5 ml. of 0.14M-sodium chloride, pH 5) prepared from calf thymus according to the method of Kay, Simmons & Dounce (1952), various quantities of solutions of the four histone fractions, and of whole histone (1 mg./ml. in 0.14M-sodium chloride, pH 5), were added. The precipitates formed were removed by centrifugation at 3000*g* for 30 min. and the extinctions of the supernatants determined at 260 m μ , from which the percentages of DNA precipitated were calculated. The resultant curves are shown in Fig. 1 (a). Nearly all the DNA is precipitated by f1 at a lower concentration of histone than with other fractions. About 5% of the DNA remains unprecipitated by excess of histone. To precipitate 1 mg. of DNA, 0.7 mg. of f1 and about 1.6 mg. of the other fractions are required.

Stoichiometric combination of histone with DNA (1 basic amino acid to each phosphate group) requires a weight ratio of histone to DNA of about 1.3–1.4. Therefore complete precipitation of DNA by f1 is brought about by about half the amount required for stoichiometric combination, whereas with f2 (a), f2 (b) and f3 more than the stoichiometric amount is required.

It is evident that the combination of f1 with DNA is of a different character from that of the

other fractions. Differences between f1 and the other fractions have also been noted in extraction experiments on the native nucleoprotein, although paradoxically in the opposite direction to what might have been expected from the above experiments. Thus f1 is preferentially extracted with weak acids, such as citric acid (Davison & Butler, 1954), or with perchloric acid (Johns & Butler, 1962).

The precipitates obtained in the above experiments are not necessarily in an equilibrium condition. Some additional experiments have been carried out under conditions in which some degree of equilibration could occur. Histone fractions (1 mg./ml.) were added to DNA (1 mg./ml.) in 2M-sodium chloride, followed by dialysis against (a) 0.14M-sodium chloride, (b) for 24 hr. against distilled water. In case (a) 0.3 mg. of histone fractions added to 1 mg. of DNA precipitated the following amounts of nucleoprotein: f1, 1.01; f2 (a), 0.88; f2 (b), 0.90; f3, 0.88 mg. In case (b), with the same quantities: f1, no precipitate; f2 (a), 0.58; f2 (b), 0.64; f3,

Fig. 1. Precipitation of DNA and RNA from solution (0.5 mg./5 ml.) by the addition of solutions of whole histone and histone fractions (1 mg./ml.). Δ , Whole histone; $\circ$, lysine-rich histones, f1; $\bullet$, intermediate histones, f2 (a); $\square$, intermediate histones, f2 (b); $\blacktriangle$, arginine-rich histones, f3.

0.48 mg. Under condition (a) it is evident that all histone fractions precipitate considerably more DNA than in Fig. 1 (a). After dialysis against water with the fractions other than f1, about half as much nucleoprotein as in (a) was precipitated.

It thus appears that, whereas the fraction f1 preferentially precipitates DNA in 0.14M-sodium chloride, the nucleohistone formed is soluble at lower ionic strengths. Complete solution is not obtained with other histone fractions. Huang, Bonner & Murray (1964) noted the complete solubility of nucleohistone formed from a lysine-rich fraction, at low ionic strengths, up to (histone:DNA) ratios of about 0.65. No data are, however, given for other fractions.

The results obtained indicate a marked difference between f1 and other histone fractions with regard to the precipitation of DNA, and this is paralleled by their effects on RNA synthesis in systems containing DNA (Barr & Butler, 1963; Huang *et al.* 1964). However, although the complexes of f1 and the other histones differ in their solubility behaviour, the ability to catalyse RNA synthesis does not appear to be influenced very much by whether they are actually present in a soluble or gelatinous form (Huang *et al.* 1964; Barr & Butler, 1963).

In view of possible interactions between RNA and deoxynucleohistones, it was decided to study the precipitation of RNA by histone fractions. The RNA used was a product of rather low molecular weight ($S = 2-3s$) prepared from the cytoplasm of rat liver by the method of Kay & Dounce (1953); the results of experiments carried out under similar conditions to those described for DNA are shown in Fig. 1 (b). There are very marked differences in the ability of the different histone fractions to precipi-

tate this RNA; f3 and f2 (a) precipitate about 80 % of the RNA at a weight ratio 2:1, whereas whole histone and f2 (b) precipitate about 50 % of the RNA, at the same ratio, and f1 is relatively ineffective. In all cases the RNA begins to redissolve with histone:RNA ratios greater than 2. These findings are no doubt influenced by the heterogeneous nature of the RNA used, and it will be necessary to examine the behaviour of homogeneous preparations of RNA of various types. An almost complete separation of DNA and RNA could be obtained by preferential precipitation of DNA under the appropriate conditions with f1.

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On the Identification of Phosphatidylethanolamine in Pigeon Breast Muscle

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Davenport (1964) concluded that the major ninhydrin-positive component of the phospholipid of pigeon breast muscle was different from phosphatidylethanolamine. He comments that it is very likely that the values for the percentage content of phosphatidylethanolamine in this muscle given by Gray & Macfarlane (1961) refer principally to this new compound. Davenport's evidence for the existence of a new lipid is derived from the properties of the water-soluble product obtained by

mild alkaline hydrolysis in conditions which presumptively give a phosphoric diester. The cephalin fraction in question, for which no percentage composition was given, amounted to 34 % of the phospholipid and had a ratio N:P about 1.6:1.0, owing to the presence of some nitrogenous impurity. The hydrolysis product had apparently a ratio N:P about 1.2:1.0. It behaved differently from glycerylphosphorylethanolamine on an ion-exchange column, and separated from this compound on

paper chromatography in one solvent, but did not separate in another solvent nor on paper electrophoresis at pH 3.2. The nature of the nitrogenous base was not examined and no evidence that it was not ethanolamine was given, apart from the above observations.

Davenport also examined by paper chromatography the alkaline-hydrolysis product of the total lipid from the longissimus dorsi muscle of the ox. He concluded that the new phospholipid was present in this muscle also, in amounts which corresponded to about 80 % of the cephalin fraction. We think this result is indicative of some artifact, for the brain and skeletal muscle of the ox were the sources for the classical isolations of ethanolamine phospholipids (Folch, 1942; Rapport, Lerner, Alonzo & Franzl, 1957) and for many years it has been accepted that ethanolamine is the main, though not always the only, nitrogenous base in animal cephalins.

The object of our earlier work (Gray & Macfarlane, 1961) was to examine the pattern of the fatty components in different phospholipids from different tissues and species; the detailed analyses by which the phospholipids had been identified were omitted but the methods used were cited. The salient points relating to pigeon breast muscle (Tables 1 and 2 in Gray & Macfarlane, 1961) are now recorded. The phospholipid fraction contained 3.7 % of P on the dry weight, with a ratio amino N:P of 0.43:1.0. The cephalin band obtained from a silicic acid column contained 38 % of the total P and the proportions amino N:fatty acid ester:P were 0.92:1.9:1.0. Paper chromatography of the intact lipid and the water-soluble product of alkaline hydrolysis indicated phosphatidylethanolamine as the main lipid component, with smaller amounts of phosphatidylserine and inositide. Estimation of ethanolamine and serine in the cephalin by the method of Axelrod, Reichenthal & Brodie (1953) gave values for these substances which accounted for the amino N and were consistent with the size of the chromatographic spots. The percentage of the total P present as ethanolamine compounds was calculated from these values.

As the method of Axelrod *et al.* (1953) is not specific for serine and ethanolamine, we have repeated the fractionation and examined the nitrogenous bases. Pigeon breast muscle (96 g.) was homogenized in 20 vol. of chloroform-methanol (2:1, v/v) at 18° and the filtered extract was washed three times with one-fifth of its volume of 0.9 % sodium chloride. The small interfacial layer of 'proteolipid' was discarded and the chloroform layer was dried with anhydrous sodium sulphate and filtered. The solvent was evaporated and the residue, about 4.0 g., was dissolved in chloroform; the solution contained 70 mg. of P, with a ratio

total N:P of 1.13:1.0. The material (54 mg. of P) was chromatographed on a silicic acid column and the eluent fractions of the cephalin band were monitored on silica-gel plates. The tail fractions (2.4 mg. of P), which visibly contained phosphatidylserine and inositide as well as the main spot, were discarded. The main fraction (15.5 mg. of P, 29 % of the total P applied) contained 3.9 % of P on dry weight; the proportions aldehyde:amino N: total N:P were 0.12:1.06:0.95:1.0. The material gave a single ninhydrin-positive spot on a silica-gel plate, and a main spot corresponding to phosphatidylethanolamine and a faint spot for phosphatidylserine on silicic acid paper.

A portion of the phospholipid (13.8 mg. of P) was hydrolysed in 3.0 ml. of chloroform, 12 ml. of ether and 15 ml. of 0.1 methanolic N-NaOH at 18° for 20 min. The mixture was brought to about pH 7.0 with 0.1 N-HCl and the fatty components were removed by extraction with ether. The aqueous solution contained 91 % of the P with a ratio N:P of 0.98:1.0; it gave ninhydrin- and phosphate-positive spots corresponding to glycerylphosphoryl-ethanolamine and -serine on paper chromatography in phenol-water-ethanol-acetic acid (50:22:3:3, by vol.) and in butan-1-ol-acetic acid-water (5:4:1, by vol.). A part was evaporated to a syrup and hydrolysed in 2 N-H₂SO₄ for 2 hr. at 100°. The solution was neutralized with barium carbonate and treated with 2 vol. of ethanol. The organic phosphate recovered from the precipitate was a monophosphoric ester by enzymic assay. The ethanolic supernatant was acidified with HCl and evaporated to dryness. The solid material gave on paper chromatography in butan-1-ol-acetic acid-water (4:1:5, by vol.) a main spot that corresponded to authentic ethanolamine hydrochloride and co-chromatographed with this substance, and a faint spot corresponding to serine. Similar results were obtained in the phenol-water-acetic acid-ethanol system.

We conclude that the main component of the cephalin fraction of pigeon breast muscle is phosphatidylethanolamine and we obtained no evidence of a new major component. We think it is possible that the discrepancies observed by Davenport (1964) were due to artifacts of the type discussed by Wren (1960), caused by the presence of nitrogenous impurities during drying, storing and processing of the phospholipid.

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The Isomer of Oxaloacetate Produced by the Action of Citrate Lyase on Citrate

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Oxaloacetic acid and its anions exist in aqueous solution as equilibrium mixtures of the keto and enol isomers, their equilibrium concentration ratios being dependent on pH (Tate, Grzybowski & Datta, 1964). The rate of attainment of keto-enol equilibrium also depends on pH as well as on temperature (Banks, 1962). At pH 7.4 and 25° equilibrium is attained almost instantaneously, but at 2° equilibration is a much slower process. The molar extinction coefficient at 280 m μ of the enol isomers ($\epsilon_{280\text{ m}\mu}$ 3600) is about 140 times as great as that of the keto isomers (Banks, 1961; Tate *et al.* 1964). Thus the observed extinction at 280 m μ ($E_{280\text{ m}\mu}$) of oxaloacetate solutions is almost completely due to their content of enol isomers. It is therefore possible to determine which isomer of oxaloacetate is produced during the enzyme-catalysed cleavage of citrate at 2° in the presence of excess of enzyme by following the production of oxaloacetate both by measurement of $E_{280\text{ m}\mu}$ and by measuring the total oxaloacetate in portions of the same solution by the borate method (Greenwood & Greenbaum, 1953). In these conditions the rate of keto-enol isomerization is less than the rate of oxaloacetate production.

The substrate-induced enzyme citrate lyase (EC 4.1.3.6) was prepared in pure form from extracts of *Aerobacter aerogenes*, NCTC 418, by a modification of the procedure of Siva Raman (1961). The reaction mixture contained 4 mM-trisodium citrate and 1 mM-MgCl₂ in a triethanolamine-HCl buffer, pH 7.4 and $I \sim 0.06$. It was kept at about 2° in an ice-water bath. The enzyme (2.5 units/ml. of reaction mixture) was added, the whole was quickly mixed and 3.0 ml. of the resultant solution was pipetted into the cuvette of an Optica CF4 recording spectrophotometer [Optica (U.K.) Ltd., Gateshead upon Tyne] fitted with a thermostatically-controlled cell holder maintained at about 2°. The increase in $E_{280\text{ m}\mu}$ was recorded against time by using an appropriate blank. During the same period portions (0.5 ml.) of the remaining solution were withdrawn at noted times and pipetted into 0.2 ml. of cold 30% (w/v) trichloroacetic acid. After centrifugation to remove the protein, the total oxaloacetate (A_T) in these solutions was determined by the borate method. The concentration of enolic oxaloacetate in the reaction mixture (A_E) was determined from the relation $A_E = E_{280\text{ m}\mu}/\epsilon_{280\text{ m}\mu}$, assuming the contribution of keto isomer to

$E_{280\text{ m}\mu}$ to be negligible. The concentration of ketonic oxaloacetate (A_K) was determined from the relation $A_K = A_T - A_E$.

The results are shown in Fig. 1; it is apparent that A_K is virtually equal to A_T for the first 1.5 min. of the reaction and it is only after this delay that A_E begins to be significant. The results therefore show that it is the keto isomer of oxaloacetate which is the one produced during the enzymic cleavage of citrate by citrate lyase at pH 7.4. Presumably ketonic oxaloacetate is also the reactive species in the synthesis of citrate by this enzyme, and in this specificity citrate lyase resembles citrate synthase

Fig. 1. Production of oxaloacetate from citrate by citrate lyase at pH 7.4 and at 2°. ●, Total oxaloacetate, A_T , determined by the borate method; ○, enolic oxaloacetate, A_E , determined from measurements of $E_{280\text{ m}\mu}$ from the relation $A_E = E_{280\text{ m}\mu}/\epsilon_{280\text{ m}\mu}$; - - -, ketonic oxaloacetate, A_K , determined from the relation $A_K = A_T - A_E$. The reaction mixture contained 4 mM-trisodium citrate and 1 mM-MgCl₂ in triethanolamine-HCl buffer, pH 7.4 and $I \sim 0.06$. Enzyme (2.5 units/ml. of reaction mixture) was added at zero time.

(EC 4.1.3.7), for which ketonic oxaloacetate is known to be the substrate (Englard, 1959). These results also suggest the possibility that it is the enol isomers that are the cause of the observed inhibition of citrate lyase by oxaloacetate (Dagley & Dawes, 1955; Bowen & Rogers, 1963).

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