and glutamic acid of casein come from the free glutamine and glutamic acid respectively of blood. Allowing for experimental error, we may conclude with certainty that over 70 % of the glutamine and glutamic acid of casein come directly from the blood. An experiment with [$^{14}$C]glucose also showed that less than 20 % of the glutamine and glutamic acid of casein were synthesized from glucose within the mammary gland (Barry, 1956). From similar experiments it may be concluded that over 70 % of the tyrosine of casein comes from the free tyrosine of blood, and is not derived from plasma proteins, or synthesized from phenylalanine within the gland (Barry, 1952). A further experiment showed that at least 50 % of the asparagine residues of casein come from the free asparagine of blood, and that less than 17 % are synthesized from glucose within the mammary gland. No significant part of the aspartic acid of casein comes from the asparagine of blood, nor is it synthesized within the gland from glucose; however, its precise origin remains unknown (Sansom & Barry, 1958). Arterio-venous differences in free serine, of up to 48 % of the arterial concentrations, were found across the mammary glands of lactating, but not dry, cows (Sheldon-Peters & Barry, 1956). These results suggest that at least part of the serine of casein comes from the free serine of blood. The source in the blood of the cystine, alanine and glycine of casein is unknown, but these amino acids make up only about 6 % by weight of the casein molecule.

From these findings it can be calculated that about half, at least, of the residues of non-essential amino acids in casein are derived directly from the blood. It would be interesting to know whether any synthesis of non-essential amino acids occurs in the mammary gland, since it is possible that only a few organs of the body can synthesize some of them, as has been suggested by Eagle, Oyama & Levy (1957). Experiments of the type described in this paper, however, will never exclude the possibility that small amounts of non-essential amino acids are synthesized within the mammary gland.

## SUMMARY

1. From an experiment with [$^{14}$C]proline it is concluded that the mammary gland of the goat takes free proline from the blood to provide at least half of the proline residues of casein.

2. From this and previous work it is calculated that at least half the residues of non-essential amino acids in casein are not synthesized in the mammary gland, but come directly from the blood stream.

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# The Reactivity of Free Amino Groups in Native and Denatured Ovalbumin Towards Fluorodinitrobenzene

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Porter (1950) showed that ovalbumin contained no *N*-terminal residue able to react with 1-fluoro-2:4-dinitrobenzene (Sanger, 1945), but subsequent work by Niu & Fraenkel-Conrat (1955) showed that it did contain a *C*-terminal residue (proline). Linderstrøm-Lang (1952) suggested that ovalbumin had one of two possible structures. Anfinsen & Redfield (1956) preferred one of these structures and considered ovalbumin to be a cyclic molecule (probably held by S–S bonds) from which protruded a peptide chain containing the *C*-terminal residue. They suggested that the terminal α-amino group was either masked sterically or chemically bound in a linkage which may be intramolecular or involve the carbohydrate moiety known to be present in ovalbumin (Neuberger, 1938).

The present work describes the effect of increasing amounts of denaturing reagents on the reactivity of $\epsilon$-$NH_2$ groups of the lysine residues of ovalbumin towards 1-fluoro-2:4-dinitrobenzene. The results indicate that the denaturation of ovalbumin is progressive and suggest that the protein contains three types of lysine residues, differing in their reactivity, or accessibility, towards 1-fluoro-2:4-dinitrobenzene. It is suggested that a structure of the type described by Anfinsen & Redfield (1956) could account for these results.

## EXPERIMENTAL

Nessler's quantitative reagent (British Drug Houses Ltd.) and 1-fluoro-2:4-dinitrobenzene (FDNB) (Light and Co., and Kodak Ltd.) were used.

*Ovalbumin.* This was prepared by the buffered ammonium sulphate method of Cole (1932). The protein was recrystallized six times and finally freed from salt by prolonged dialysis. Analysis of the product (dry and ash-free) gave: total N, 15·72% [cf. 15·76% (Tristram, 1949)]; amide-N, 1·04% [cf. 1·04% (Rees, 1946)].

The crystalline ovalbumin was examined by starch-gel electrophoresis (Smithies, 1955) in borate buffer, pH 8·6 (0·03 M-$H_3BO_3$; 0·012 M-NaOH). Three distinct bands were clearly distinguishable with only slight differences in electrophoretic mobility. The fractions appeared in approximately the proportions given for $A_1$, $A_2$ and $A_3$ (79, 15 and 6% respectively) by Cann (1949). Since the fractions $A_1$, $A_2$ and $A_3$ are said to contain two, one and no phosphate groups respectively, $A_1$ should have the greatest mobility at pH 8·6. It was observed that the leading component on starch-gel electrophoresis formed the major fraction of the ovalbumin.

Whole egg white was examined under the same conditions, when it was observed that the albumin fraction became subdivided into three components. These components were eluted together and examined electrophoretically alongside ovalbumin. The eluted fraction separated into three components whose mobilities were identical with those of $A_1$, $A_2$ and $A_3$ respectively of the albumin.

*Preparation of 2:4-dinitrophenyl-ovalbumin.* The reaction with FDNB is normally carried out in the presence of ethanol (65%, v/v), and considerable denaturation might be expected to occur. A milder reaction may be used in which FDNB is allowed to react at 37° in aq. 8% (w/v) $NaHCO_3$ in the absence of ethanol (Courts, 1954). Under these latter conditions no denaturation takes place.

In the present work four types of reaction with FDNB were carried out (see Table 2): (*a*) in the absence of ethanol without prior denaturation (cf. Courts, 1954); (*b*) in the presence of ethanol without prior denaturation (cf. Sanger, 1945); (*c*) after prior denaturation and removal of the denaturant (ethanol) by aeration at low temperature; (*d*) in the presence of denaturants other than ethanol (guanidine, urea, KI, KSCN). [In all cases the reaction was stopped after a given time by acidification followed by precipitation of the 2:4-dinitrophenyl (DNP)-protein with ethanol.] The product was washed exhaustively with ethanol and ether to remove all traces of FDNB and dried *in vacuo*.

*Determination of protein in 2:4-dinitrophenyl derivative by estimation of amide nitrogen.* A modified form of the method of Bailey (1937) was used. Five samples of varying weight of DNP-protein (0·5–10·0 mg.) were hydrolysed with 2 N-HCl for 3 hr. under reflux. The hydrolysates were made alkaline to thymolphthalein and made to 100 ml. with glass-distilled water. The $NH_3$ was removed by micro-Kjeldahl distillation (6 min.) and collected in 2 ml. of 0·01 N-HCl, the final volume being 25 ml. Duplicate 10 ml. portions of the distillate were treated with 2·5 ml. of Nessler's reagent and the extinction was read on an EEL photoelectric colorimeter (Ilford filter no. 303, maximum transmission at 480 m$\mu$). The results were plotted as in Fig. 1*b* and amide N was obtained from the calibration curve obtained by putting $(NH_4)_2SO_4$ through the same procedure (Fig. 1*a*).

*Example.* The $NH_3$ from 7·6 mg. of DNP-protein gave an extinction (corrected for blank) of 15·1 (Fig. 1*b*). From the standard curve (Fig. 1*a*) this is equivalent to 28·5 $\mu$g. of N. Thus the DNP-protein contains 0·94 g. of amide N/100 g. of dry ash-free DNP-protein. Crystalline ovalbumin contains 1·04% of amide N; hence the DNP-albumin contains 90·5% of ovalbumin.

*Analysis of 2:4-dinitrophenyl-ovalbumin.* Five samples of each DNP-ovalbumin were hydrolysed with 6 N-HCl (glass-distilled) for 18 hr. After cooling, each hydrolysate was extracted four times with 20 ml. of ether (80 ml. in all). The ether-soluble fractions were pooled and concentrated. Chromatographic analysis on buffered paper (Blackburn & Lowther, 1951) and on Celite columns (Courts, 1954) demonstrated the complete absence of any $\alpha$-DNP-amino acids. 2:4-Dinitrophenol was detected in all cases and its presence was confirmed by measurement of its ultraviolet-absorption spectrum in dilute acid ($\lambda_{max.}$ 260 m$\mu$) and alkali ($\lambda_{max.}$ 345 m$\mu$).

Chromatographic analysis on silica gel with butan-2-one–ether as mobile phase (Sanger, 1945), and on Celite with the same solvent system (Courts, 1954), showed that all the lysine residues were linked as $\alpha$-peptides, since only $\epsilon$-DNP-lysine was found in acid hydrolysates of fully substituted DNP-ovalbumin ($\alpha$-DNP-lysine and $\epsilon$-DNP-lysine have $R_F$ values of 0·11 and 0·20 respectively, with the above systems).

The water-soluble fractions were concentrated by vacuum distillation until all traces of HCl had been removed. Each residue was dissolved in aq. 1% (w/v)

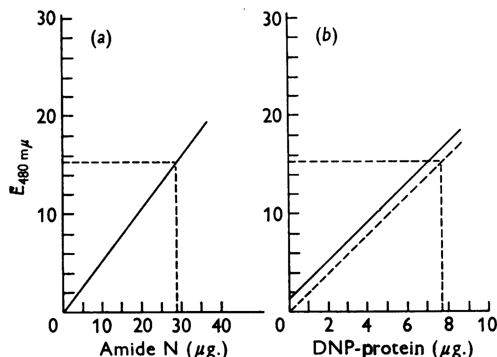


Fig. 1. Estimation of amide nitrogen. For details see text. (*a*) Standard curve. (*b*) DNP-protein: —, uncorrected readings; - - - -, corrected for blank reading.

Table 1. *Reaction of ovalbumin with 1-fluoro-2:4-dinitrobenzene*

The total number of $\epsilon$-lysine groups/molecule is 20 (Tristram, 1949).

| | Prior denaturation | | | | Reaction with FDNB (18 hr.) | | | |
|---|---|---|---|---|---|---|---|---|
| Expt. | Reagent | Concn. (M) | Time (hr.) | Temp. | Ethanol (%, v/v) | Temp. | $NaHCO_3$ (%) | No. of $\epsilon$-DNP-lysine groups/mol. (45 000) |
| 1 | — | — | — | — | — | 37° | 5 | 3 |
| 2 | — | — | — | — | 35 | 20 | 2 | 9 |
| 3 | — | — | — | — | 50 | 20 | 2 | 12 |
| 4 | — | — | — | — | 60 | 20 | 2 | 20 |
| 5 | Urea | 8 | 18 | 37° | — | 37 | 5 | 20 |
| 6* | Urea | 8 | 18 | 37 | — | 37 | 5 | 20 |
| 7† | Urea | 8 | 18 | 37 | — | 37 | 5 | 3 |
| 8 | Guanidine | 0·5 | 4 | 37 | — | 37 | 5 | 7 |
| 9 | Guanidine | 0·5 | 0·5 | 37 | 50 | 20 | 2 | 11 |
| 10 | Guanidine | 0·75 | 18 | 37 | — | 37 | 5 | 20 |
| 11 | KI | 2·5 | 18 | 37 | — | 37 | 5 | 11 |
| 12 | KSCN | 2·5 | 18 | 37 | — | 37 | 5 | 12 |

* Preparation no. 1 after reaction with FDNB (three DNP groups) was used in place of crystalline ovalbumin.
† M-Glucose was added as an inhibitor of denaturation. Reaction with FDNB was allowed to proceed for 48 hr.

Table 2. *Progressive denaturation of ovalbumin with ethanol*

Denaturation with ethanol was allowed to proceed for 18 hr. at 37°. After removal of ethanol, the reaction with FDNB was carried out at 37° for 18 hr. in the presence of 5% $NaHCO_3$ (cf. Courts, 1954).

| Ethanol (%, v/v) | No. of $\epsilon$-DNP-lysine groups/mol. (45 000) | Percentage of total groups reacting |
|---|---|---|
| 0 | 3 | 15 |
| 10 | 5 | 25 |
| 20 | 6 | 30 |
| 30 | 8 | 40 |
| 40 | 10 | 50 |
| 50 | 12 | 60 |
| 55 | 20 | 100 |
| 60 | 20 | 100 |
| 70 | 20 | 100 |

$NaHCO_3$ and the absorption measured at 360 m$\mu$. The quantity of $\epsilon$-DNP-lysine present in each aqueous fraction was calculated from a calibration curve obtained by submitting mixtures of $\epsilon$-DNP-lysine and ovalbumin to hydrolysis under similar conditions. Hydrolysis with 6N-HCl for 18 hr. led to the degradation of 8% of $\epsilon$-DNP-lysine and it was therefore assumed that the same loss occurred when $\epsilon$-DNP-lysine was liberated from peptide linkage.

## RESULTS

Ovalbumin (mol.wt. 45 000) contains 20 lysine residues/molecule (Tristram, 1949), all of which can react with FDNB. From the analytical data (Table 1) three main conclusions are apparent: (1) ovalbumin contains no *N*-terminal residue; (2) all the lysine residues have free $\epsilon$-$NH_2$ groups, only $\epsilon$-DNP-lysine being isolated after acid hydrolysis; (3) the extent of the reaction between FDNB and ovalbumin is changed by denaturation (see Expts. 1, 6 and 7.) Complete denaturation (Expt. 5) exposes an additional 17 groups which are not reactive in native ovalbumin (cf. Expt. 1). M-Glucose completely inhibits denaturation (cf. Expt. 7) by urea. [Warner (1954) suggested that ovalbumin was protected from denaturation by sugars, including glucose.]

Ovalbumin contains lysine groups which fall into three classes with respect to their reactivity or availability toward FDNB: (*a*) three readily available residues; (*b*) nine residues reacting only after partial denaturation, and these independently of each other (cf. Expts. 2, 3, 8, 9 and 11); (*c*) eight residues which react simultaneously after denaturation has reached a definite stage (Expts. 4, 5, 6 and 10). Numerous attempts, with no success, have been made to vary conditions so as to obtain a number of $\epsilon$-DNP-lysine groups intermediate between 12 and 20.

Similar results were obtained in the serial denaturation of ovalbumin with ethanol (Table 2).

## DISCUSSION

If the *N*-terminal residue itself is involved in an intramolecular link or is masked, as are the final eight residues of lysine, it would be reasonable to expect that the *N*-terminal group becomes exposed at the same time as these lysine residues. No trace of any $\alpha$-DNP-amino acid was found, and the terminal amino group is thought to be masked chemically by intramolecular linkage or by

involvement with the carbohydrate moiety (cf. Porter, 1950). Although the acid lability of certain α-DNP-amino acids cannot be ignored (e.g. proline, Porter & Sanger, 1948), no evidence has been obtained for the presence of even traces of α-DNP-amino acids. Bailey (1951) could find only a trace of α-DNP-residue (1 mole/$10^6$ g. of protein) in four-times-recrystallized ovalbumin.

The availability of lysine residues towards FDNB suggests that the native protein molecule is in the form of a coiled loop, as suggested by Anfinsen & Redfield (1956). The three types of lysine residue with differing reactivity towards FDNB might be accounted for in the following manner: (*a*) three ε-$NH_2$ groups which are in easily accessible sites in the native molecule; (*b*) a further nine groups which cannot react in the native state for steric reasons. As denaturation proceeds the molecule uncoils and these groups become progressively available for substitution. This would indicate that denaturation is a stepwise process, at least with ovalbumin, rather than the all-or-nothing reaction envisaged by Mirsky & Anson (1935); (*c*) as denaturation proceeds a point is reached (50–55% ethanol; 0·5–0·75M-guanidine) when all the remaining ε-$NH_2$ groups interact. This may be an indication of the existence of a cyclic portion of the molecule or that the remaining ε-$NH_2$ groups are in fairly close sequence. It has not been found possible to substitute a number of ε-lysine $NH_2$ groups intermediate between 12 and 20. Thus whereas 50% ethanol exposes 12 groups to reaction, 55% ethanol brings all the 20 groups into a reactive state.

## SUMMARY

1. The reaction between ovalbumin and 1-fluoro-2:4-dinitrobenzene has been studied under various conditions.

2. Progressive denaturation before reaction enables three types of ε-amino groups with different affinities for 1-fluoro-2:4-dinitrobenzene to be detected.

3. Glucose inhibits denaturation by urea and prevents the substitution of ε-amino groups beyond the minimal three groups.

4. Ovalbumin contains no free terminal amino group.

5. All the lysine residues are bound in α-peptide bonds.

6. The significance of these results is discussed.

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# Properties and Partial Purification of Kynureninase

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The work to be described originated in a general study of pyridoxal phosphate-linked enzymes. Jakoby & Bonner (1953*a*) obtained kynureninase from *Neurospora crassa* (Em 5256A), studied its properties and claimed to have purified it 70- to 100-fold. This work is being reported as some of their results could not be corroborated. Its special purpose was to study the following problems: (1) To determine the optimum conditions for kynureninase activity. (2) To corroborate the general mechanism of action of pyridoxal phosphate-linked enzymes proposed by Metzler, Ikawa & Snell (1954). To do this it is essential to establish that metal ions are cofactors of these enzymes;

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