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Fluorescence associated with proteins

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NOTE TO USERS

FLUORESCENCE ASSOCIATED WITH PROTEINS

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I. INTRODUCTION

The manufacture of cheese, tanning of hides and the preparation of glue, as well as the coagulation of proteins during cooking processes, were known to the ancients. These applications of protein chemistry were useful because of the chance observations of many people.

The wide distribution of proteins and their indispensability to the living organism makes them important objects of chemical research which has caused the development of protein chemistry. The initial development came about partly by chance observations and partly by research studies. The earlier protein chemists dealt entirely with the application of extraction methods to biological material. They were able to apply extractions with water, dilute acids and alkali, as well as with neutral salt solutions to both plant and animal tissues for the removal of proteins. These methods had already been discovered by the early descriptive chemists.

Following the isolation of the proteins, studies of their elementary composition led Liebig to conclude that proteins differed in state but not in substance. He recognized four kinds of proteins and believed that proteins with the same elementary compositions were identical

whether they came from plant or animal tissue. However, with the development of more accurate methods of elementary analysis, Liebig's hypothesis was short lived.

Modern protein chemistry dates from the year 1820 when Braconnot isolated glycine from the hydrolysis of gelatin with sulfuric acid. He believed the crystalline material obtained to be a sugar and it was several years after its discovery that glycine was found to contain nitrogen. The discovery of other amino acids resulted from observations on the crystalline substances obtained from the fermentation of cheese, analysis of urinary calculi, fusion of proteins with potassium hydroxide, and studies on the extracts of plant and animal tissues. Some amino acids have been discovered as a result of the realization that all of the protein nitrogen could not be accounted for by the known amino acids. Recently, others have been discovered as a result of nutritional investigations.

The work of Fischer and his pupils did a great deal to advance the peptid concept of the protein molecule as well as develop better methods of attack on the analysis of the complex mixtures obtained by the hydrolysis of proteins. Many of his ideas and technics are being used and developed at the present time.

With the advent of proteolytic enzyme methods of attack on the structure of the protein molecule, new advances were

made. Since the fragments obtained from the enzyme diges-
tion of proteins were difficult to purify and characterize,
the structure of proteins seemed more complex. However,
by means of careful research and study of the action of
proteolytic enzymes on synthetic peptids and polypeptids,
new advances have been made.

By developing physico-chemical methods and applying
them to protein chemistry, Loeb and many others have intro-
duced concepts which are useful. They have also elucidated
the behavior of proteins in solution by these methods.

Although the fluorescence of proteins in nature was
noted as early as 1855, the phenomenon has not been ex-
plained nor have research chemists applied this phenomenon
to protein chemistry. Observations have been made as to
the color of fluorescence of proteins but the effects of
protein hydrolysis on the fluorescence have not been
studied.

The re-emission of light by solids, liquids and gases
under the stimulating influence of incident radiation is
called luminescence. If the re-emission stops practically
at once when the exciting light is shut off it is called
fluorescence. In solids, however, this re-emission may
continue, even for hours, after the exciting radiation has
been cut off. This phenomenon is known as phosphorescence.

Fluorescence has been noted in numerous biological materials but very few studies have been made to determine the specific constituents present in these materials which emit the fluorescent light. The present investigation was undertaken to determine whether or not the fluorescence of proteins could be used in protein chemistry. More specifically, the objectives were: first, to determine whether or not the fluorescence of proteins is due to occluded or adsorbed material; second, to determine the effect of protein hydrolysis by various methods on the fluorescence of the proteins; and third, to study the products of protein hydrolysis and determine which of the constituents are fluorescent.

II. REVIEW OF LITERATURE

The literature on the fluorescence of biological materials is too voluminous to be completely reviewed in this thesis. Since all proteins studied are fluorescent in the blue-green region of the spectrum, only those biological materials which are difficult to separate from proteins and which fluoresce in the blue-green will be reviewed. Since the porphyrins, chlorophyll and alkaloids can be obtained free from protein, they will not be included in this review. Likewise, all lipoids will be omitted.

A. Urine and Blood Serum

The fluorescence of urine has been known for over a hundred years, yet the substances present in it which cause this phenomenon have not as yet been fully identified. Jaffe (40) published an article in 1869 in which he stated that two other investigations previously had shown that the blue fluorescence of urine faded in acid solutions and returned when alkali was added. These early investigators gave no explanation of the fluorescence or its chemical behavior. Jaffe was isolating urinary pigments when he noticed the fluorescence of urine and made several

unsuccessful attempts to isolate the causative substance.
In addition to the behavior of the fluorescent material
with acids and bases as mentioned above, he found it to be
somewhat soluble in chloroform. He also found that the
filtrates of urine, after the removal of creatine by zinc
salts, were very fluorescent and concluded that it was due
to the presence of urobilin whose fluorescence was enhanced
by the presence of the added zinc salts.

Langecker (54) studied the urinary pigments and pre-
pared a series of fluorescent salts of hematoporphyrin.
Since the fluorescence of these compounds was not like
that of urine, he concluded that urochrome, uroerythrin,
urobilin, and porphyrin were not responsible for the
urinary fluorescence.

Hadjioloff and Kresteff (30) found that the fresh
urines of all vertebrates were fluorescent to varying de-
grees and colors dependent on the species. When these
urines were dried or partially decomposed by heat, they
were more intensely fluorescent and always fluoresced in
the blue region of the spectrum when exposed to ultra-
violet light. The urines could be filtered through porce-
lain filters without destroying the fluorescence, but
treatment with animal charcoal always removed it by adsorp-
tion. The fluorescence persisted after precipitation of
urobilin or urochrome, and bacterial or enzyme action did

not affect it. In a further study of this phenomenon, these same authors (31) found that the fluorescence was not due to inorganic substances nor to urea, uric acid, hippuric acid, xanthine, nor guanine. They found that only uric acid, when partially calcined (charred) and dissolved in water, gave a blue fluorescence comparable to that of urine. Complete calcination destroyed all fluorescence of both urine and uric acid. From the extraction of partially charred urine with water, they obtained a yellow substance which was soluble in water or alcohol and less soluble in glycerine; it fluoresced in all of these solvents. The addition of alkali caused the blue fluorescence to become more green. They claim this reaction is a more sensitive test for uric acid than the murexide test.

Wassink (85) studied the blue fluorescence of urine in relation to health and disease. Although he could find no correlation between the incidence of cancer or other diseases and the amount of fluorescence in the patient's urine, he was able to show that the fluorescence could be increased. He found that synthetic indican was not fluorescent and the amount of fluorescence of the urine was not related to the indican content. He could increase the amount of fluorescence in the urine by feeding the patient small amounts of indole. He tried to

isolate the fluorescing compounds from urine, but with no
success. However, he was able to show that the substance
was adsorbed on charcoal from acid solutions and was not
salted out by full saturation with ammonium sulfate.
Since the fluorescence spectrum of the urine corresponded
to the fluorescent spectrum of mono- and diacetyl indoxyl
(synthetic), he concluded that these compounds were re-
sponsible for the urinary fluorescence. He also found
that those urines giving abnormally high values for
urobilin fluoresced green rather than the usual blue-
green.

These conclusions of Wassink were corroborated by
von dem Borne (3) who claimed, in addition, that when the
amorphous fluorescent material from urine was rubbed into
the skin of mice it produced cancer.

Koschara (47) isolated a yellow pigment, which he
called uropterin, from the urines of men, dogs, horses,
and cattle. It was found to be identical, in all its
known properties, to the xanthopterin obtained from the
wings of yellow butterflies and from wasps. It occurs in
urine to the extent of about one part per million and
fluoresces in the blue region of the spectrum. Koschara
showed that the blue fluorescence of this substance was
strongest at pH values from 7 to 11, that it changed to
a greenish fluorescence when the acidity was changed to

pH 4, and that at stronger acidities it was non-fluorescent. At alkalinities above pH 11 and in sodium carbonate solutions, uropterin fluoresced green. The color of the pigment also changed with change of acidity, being colorless in strong mineral acids, yellow in acetic acid, green-blue at pH 4 to pH 11, and changing back to yellow in sodium hydroxide solutions. He claimed that it could not be lumichrome since its fluorescence disappeared in strong mineral acids and changed to green in solutions of sodium carbonate. It was not thiochrome because it gave a positive murexide test.

Uropterin was readily adsorbed from acid solutions onto various acid silicates (Fuller's earth, Floridin, etc.) and was eluted from them with pyridine-water or pyridine-alcohol mixtures. It was incompletely extracted from aqueous solutions by butyl or amyl alcohol. It formed barium and silver salts by means of which it was purified.

In a later publication, Koschara, von der Siepen and Aldred (48) report that only about one-half to one-fourth of the total fluorescence of the urine is due to uropterin and that the other blue-fluorescent substances have not been identified. They also showed a relationship between the amount of uropterin in the urine and the amount of nitrogen metabolism of the body, suggesting that uropterin is an indicator of protein catabolism.

Sterling-Okuniewski and Penska (75) examined a large number of blood samples for fluorescence of the blood serum in ultra-violet light of wavelengths shorter than 3650 $\overset{o}{A}$. They state that a large variety of diseases caused no change in the fluorescence of the blood serum, and all samples showed a uniform blue fluorescence which was stable for months if the sera were kept sterile. The few pleural exudates examined by them also showed this same blue fluorescence which, like that of blood serum, was stable to irradiation by short ultra-violet light or by X-rays and was not affected by the addition of cholesterol. They also found the blood serum of animals to exhibit the same properties as human blood serum with regards to its fluorescence and stability.

Brunner (5) found that green fluorescence of blood serum occurred only when there was an excessive amount of urobilin in the blood.

B. Vitamins and Fluorescence

It is well known that lactoflavin fluoresces yellow-green and when irradiated in alkaline solution it is converted into chloroform-soluble blue-fluorescing lumiflavin. Lumiflavin was discovered by Warburg and Christian (83) (84) and Kuhn et al. (52) in 1932 and was synthesized two years later by Kuhn and Reinemund (49). According to Kuhn

and Rudy (50) it is formed by irradiation of lactoflavin only in alkaline solution. It can be extracted from acidified aqueous solutions by chloroform and like lacto-flavin yields urea when boiled with barium hydroxide solution.

Lumichrome, the other photolytic decomposition product of lactoflavin, was discovered by Karrer et al. (44) who also proved its structure. It is formed from lactoflavin by irradiation in neutral or slightly acid solution (50). It is less soluble in chloroform than lumiflavin but also gives urea when boiled with barium hydroxide.

Thiochrome, the blue-fluorescent oxidation product of vitamin B_1, was discovered by Peters (45) (60) (61) and isolated by Kuhn and his associates the same year (53). It is destroyed by boiling with 2 $\underline{N}$ sodium hydroxide solution, is soluble in chloroform and in amyl alcohol and reversibly reduced by sodium hydrosulfite. Its fluorescence is greatly diminished at acidities below pH 5, and it is irreversibly oxidized by irradiation in alkaline solutions (51) (53).

The extent to which these three blue-fluorescent decomposition products of vitamins occur in nature is not known; however, Cohen (8) has described a blue-fluorescent substance obtained from carrots. Since it can be reversibly reduced with sodium hydrosulfite, is soluble in chloroform

and its fluorescence is diminished in acid solutions, it is probably thiochrome. This, however, has not been proved. Fischer (17) has recently shown that there is no thiochrome in the lens of the eye of cattle to account for the blue fluorescence always present in eye lenses.

C. Fluorescence of Animal Tissues

As early as 1855 fluorescence was known to be associated with the lenses of the eyes of several species of animals. Bence Jones (42) gave a review of the references to fluorescence of the eye lens previous to 1866. He stated that in 1845 Brucke found the lens of the human eye was able to absorb blue wavelengths of light. Ten years later Helmholtz found the lens to be fluorescent. Jones also stated that Regnauld, in 1858, found the cornea to be fluorescent and in 1859 Setchenow, a pupil of Helmholtz, took the spectrum of the fluorescence of the eye lens. He found that it emitted light in the blue-green region of the spectrum when irradiated with ultra-violet light.

Bence Jones found that many animal tissues, both living and dead, fluoresced in the blue region of the spectrum. According to him the fluorescence of the eyes of man, guinea pig and of bullocks did not disappear when the excised lens was kept in glycerine for months. He also found that the fluorescent substance could be extracted

from the tissues by heating with dilute acids. Like quinine, it was extracted by ether only from alkaline solutions. It was not destroyed by acid permanganate; but, like quinine, its fluorescence disappeared when treated with alkaline permanganate. Since it was also precipitated by alkaloidal reagents, he called it "animal quinoidine."

Stübel (76) has made a careful survey of the fluorescence occurring in animal tissues. Using light of wavelengths 3000-4000 $\overset{o}{A}$ he found, in addition to the lens and retina, the skins of animals fluoresced bluish-white and the less pigmentation of the hair and skin the more pronounced was their fluorescence. He found that all tissues fluoresced to some extent, but teeth, hair, nails and fats were extremely brilliant. He was the first to report that such isolated proteins as albumin, vitellin, and elastin, all showed bright blue fluorescence. Although peptone fluoresced greenish-blue in ultra-violet light, he found gelatine, gluten, keratin, asparagin, and tyrosine fluoresced blue. Although the fluorescence of the lens of the eye was found to be the strongest of the body, the blood and blood pigments absorbed ultra-violet light but gave no fluorescence.

Kinnersley, Peters, and Squires (46) examined many plant and animal tissues in ultra-violet light of wavelengths 3000-4000 $\overset{o}{A}$ for blue fluorescence. They divided

the fluorescent substances into two types; one was soluble in ether-alcohol solution, or was made so by treatment with alcoholic potassium hydroxide; the other type, such as occurred in skin, gelatin and the lens of the eye, could not be extracted. They were not able to isolate any of the fluorescent substances in pure form. They list the following substances as being non-fluorescent: Trypto-phane, cystine, leucine, histidine, tyrosine, glycine, and creatine. Allantoin, caffeine, hypoxanthine, uric acid, creatinine, xanthine, alloxan, parabanic acid, guanine, urea, guanidine, hippuric acid, several choles-terol derivatives, several carbohydrates, ox blood plasma, ox blood serum, and egg albumin were also non-fluorescent.

Guano and urine both contain two blue-fluorescent substances which can be separated, according to Kinnersley, Peters, and Squires (46), by fractional extraction from aqueous solutions with ether-alcohol mixtures. The ether-alcohol soluble portion, on evaporation of the solvent, gives a reddish-brown residue which fluoresces blue when diluted with aqueous alcohol solution. There was more of the aqueous layer fraction in urine than in guano. When treated with alcoholic potassium hydroxide, the ether-soluble fraction from urine or guano gives a greenish-blue fluorescence which changes to blue on neutralization with acid. They also found that bile gave a deep violet-

fluorescing acetone extract which was quite unlike that
of other animal products or tissue extracts.

These same authors also obtained blue-fluorescing
substances from sheep liver and milk by extracting them
with chloroform. White hen feathers when treated with
acid-alcohol, followed by chloroform extraction, also gave
a blue fluorescence in the chloroform layer.

Their investigations on the fluorescence of gelatin,
skin, and lens of the eye showed that these materials were
quite different from other animal tissue, or animal products,
in regard to the properties of the causative agent of their
fluorescence. The fluorescence of gelatin, skin and eye
lens was not affected by boiling with dilute acids, but
disappeared after treatment with alcoholic potassium hy-
droxide. They could in no way obtain ether-soluble extracts
which were blue-fluorescent. They obtained evidence that
the fluorescence of skin, gelatin and lens was not due to
the presence of porphyrins, and that it was stable to oxi-
dation.

They also found that yeast vitamin concentrates fluo-
resced blue but in a later publication (60) stated that
this could have been due to the oxidation products of
vitamin B_1 and lactoflavin. However, they gave no experi-
mental proof for this.

In 1935, von Euler, Adler and Brandt (14) (15) extracted a blue-fluorescent substance from the corpus luteum of the cow. This blue-fluorescent substance did not fade in alkaline solution nor in strong acids even at temperatures up to 100°C. Fat and carotene were removed from the corpus luteum by ether extraction and the lumiflavin by chloroform extraction. They were able to separate, by adsorption on aluminum oxide, the remaining corpus luteum solution into four chromatographic zones: 1 - flavin; 2 - a brown-colored substance; 3 - a green zone; and 4 - the blue-fluorescent compound. This latter was removed from the aluminum oxide by an alkaline elution reagent and was obtained as a yellow-colored solution with blue fluorescence. A photograph of the spectrum of this fluorescence showed a broad band from 4040-4360 Å with a center at about 4200 Å. It was only slightly reduced by zinc in acid solution or by sodium hydrosulfite in alkaline solution. It became more greenish fluorescent when irradiated for an hour but did not change into lumichrome. They indicated that the substance might be an alloxazin related compound.

Among the lower forms of animal life known as echinoderms, Bierry and Gouzon (2) found a green-fluorescent pigment in Holoturis nigra. This pigment was soluble in alcohol and was very sensitive to both acids and bases.

Although its fluorescence was irreversibly destroyed by
either acids or bases, it was stable to irradiation by
sunlight in neutral solution. Its fluorescent spectrum
ranged from 4240 $\overset{o}{A}$ to 6400 $\overset{o}{A}$ with its axis at 5320 $\overset{o}{A}$.

Fontaine and Busnel (18), in 1938, reported the
extraction of a blue-fluorescent substance from the skin
of fish. (The blue fluorescence of fish skin had pre-
viously been noticed by Hadjioloff and Kresteff (32).)
This substance had the same solubility as lactoflavin but
fluoresced blue rather than greenish-yellow. It was in-
soluble in chloroform and therefore was not lumiflavin
nor thiochrome. They also state that it was not lumichrome.
In a second publication (19) Fontaine and Busnel maintain
that this blue-fluorescent substance is closely related
to or associated with melanin. It is localized in the
melanophores of the fish skin or is in immediate contact
with them. In some species of fish they could find no
blue-fluorescent substance, but instead there was an
extraordinary amount of melanin in the skin. This extract-
able material is non-fluorescent in the skin and is liberated
by dilute acid. When liberated, it is fluorescent in ultra-
violet light; it is reversibly reduced by sodium hydro-
sulfite and oxidized again by the air. Its fluorescence
is somewhat diminished by strong acids or bases but not by
acetic acid. These authors also found a blue-fluorescent

substance in the skin of teleostans (20). They believe
this latter material to be the same as the one extracted
from the skin and scales of fish. They were able to sepa-
rate it from flavin by chromatographic adsorption, but re-
port that it has flavin-like growth-promoting properties
when fed to rats on a flavin-deficient diet (67).

In 1939, Gourévitch (29) found that the skin of frog
tadpoles contained a green-fluorescent substance when they
were very young and after a certain size and age of the
tadpole had been reached the green fluorescence turned to
blue. This blue-fluorescent material was extractable with
methyl alcohol and was reduced with sodium hydrosulfite.
It was dialyzable through collodion. From its properties
he concluded that it was similar to and probably identical
with the blue-fluorescent material from the eyes of crus-
tacea.

Wels and Jokisch (87) (88) found that, under the in-
fluence of strong ultra-violet light and in the presence
of nitrogen and in the absence of oxygen, the tissue showed
a strong blue fluorescence in place of the blue-green ob-
tained in the presence of oxygen. They assumed that the
fluorescence was due to decomposition products of the pro-
teins but did not isolate them. They also showed that long
exposures to X-rays produced the same amount of fluorescence
as obtained with ultra-violet irradiation. In tissue cells,

after irradiation with ultra-violet light, the nucleus appeared much more fluorescent than the surrounding protoplasm while with X-ray irradiations, the whole cell became fluorescent.

Von Sepibus (74) examined a great number of lenses from human eyes and found that their fluorescence was more intense in older persons and the color of fluorescence changed from blue to yellow with increasing age. Schanz (69) found that the fluorescence of the lens was like that of egg albumin in that it could not be extracted by organic solvents.

Von Euler, Hellstrom and Adler (16), although more concerned with the fluorescence of the retina of the eye, found the lenses of the eyes of humans, fish, dogs, rats, and cattle to be fluorescent.

Fischer (17) found that the lens of the ox contained about 0.001 microgram of vitamin B_1, but contained no thiochrome to account for the blue fluorescence.

Hess (38) found that the eyes of insects and crabs are fluorescent and believes this is important in relation to heliotropism in these organisms.

In 1938, Gourévitch (26) began a study of the blue-fluorescent substance present in the eyes of fish and crustacea. The fluorescence could be extracted by methyl alcohol and was reversibly reduced by sodium hydrosulfite.

It was unchanged by irradiation in alkaline solution. He also found (27) that the fluorescent color varied with the pH. On acidification, the blue fluorescence became more violet and the intensity decreased very considerably at pH 2 and disappeared at lower pH values. In strong alkali the fluorescence is more green, changing to blue when the alkalinity decreases to pH 8 or 9. It is still fluorescent at alkalinities above pH 13. This series of fluorescent color changes is completely reversible by changing the pH. The fluorescence is destroyed by acid permanganate. Gourévitch also found that the fluorescent material from the eyes of lobsters gave no fluorescence color changes with changing pH values but remained blue. It was reversibly reduced by sodium hydrosulfite solutions and destroyed by acid permanganate. It differed from the fluorescent material from fish eyes only in its behavior toward changes in pH.

Von Euler and Adler (13) reported the discovery of a blue-fluorescent substance in the retina of fish eyes which could be extracted with 60 per cent aqueous alcohol solution. They found it to be dialyzable; it was adsorbed by the same acid silicates which adsorb flavins, and was eluted by the same reagents that would elute the flavins. It differed from synthetic alloxazine derivatives in that it was not destroyed by heating to 60-80°C. in 0.1 $\underline{N}$

potassium hydroxide for ten hours. Its fluorescence was diminished at pH values around 3.5 to 3.0 and disappeared altogether in strong mineral acids. On neutralization the fluorescence returned. It was not soluble in chloroform but on irradiation gave a substance resembling lumichrome. The blue-fluorescent substance was not reduced by sodium hydrosulfite and therefore it was not thiochrome. They also showed that it was not identical to lumiflavin.

Wald (82) noted the blue fluorescence of the bull-frog retina but did not characterize nor isolate the causative agent.

Brunner (4) showed that the visual purple from the retinas of animals could be divided into the following components: Cholesterol phosphatid, β-carotene, vitamin A, lactoflavin, lumiflavin, lumichrome, creatine, ascorbic acid, and an unidentified chloroform-soluble blue-fluorescent compound.

D. Fluorescence of Isolated Proteins

As early as 1911, Stübel (76) demonstrated the fluorescence of isolated proteins. He stated that they all fluoresced with a uniform blue color with the exception of peptone which fluoresced blue-green. Harden and Norris (34), O'Meara (58), and Roche (68) have all studied the fluorescence and colored products produced when diacetyl

reacts with proteins in the presence of strong alkali. They have shown that the pink color produced with diacetyl is due to a reaction between guanidine groups present in the protein and the added diacetyl, while the green fluorescence is dependent on the degradation products, having the structure of tri- or dipeptides, in combination with this red coloring matter produced from the guanidine groups. The fluorescence disappeared in the presence of very strong alkali and returned on neutralization. The fluorescence cannot be produced by the reaction of completely hydrolyzed proteins with diacetyl.

In 1928 Wels and Jokisch (86), using filtered ultraviolet light, found that irradiation of proteins increased their fluorescence as measured by the intensity of the emitted light before and after irradiation. The fluorescence increased with increased irradiation with ultraviolet light, but there was only a small increase when X-rays were used to irradiate the proteins. Infra-red rays and simple heat treatment on a water bath were only slightly effective in increasing the fluorescence. The protein decomposition products were more fluorescent if irradiated in the presence of oxygen than in its absence. Euglobulin was more fluorescent when irradiated in alkaline solution than when irradiated in acid. They attribute the increased fluorescence to the photolytic decomposition of

the proteins to produce fluorescent products of unknown composition.

Vlès (79) used water pastes of crystalline amino acids or proteins to study their fluorescence. He found many proteins to be excited to fluorescence by a large part of the ultra-violet light from 2400 or 2500 $\overset{o}{A}$ to the visible region. He noted that gelatin and serum-albumin were the most fluorescent while casein, edestin, and excelsin were less intense. Dried blood serum was extremely fluorescent; the rest of the blood components were non-fluorescent. Of the amino acids, histidine and arginine were found to be the most fluorescent while alanine, proline, tyrosine, and d-glutamic acid were very weakly so. Glycine, l-cystine and l-leucine were not fluorescent. Vles also noted that the fluorescent light from gelatin was not polarized when examined with Nicol prisms.

Pringsheim and Gerngross (64), using ultra-violet light of wavelength 3660 $\overset{o}{A}$, showed that gelatin and derived proteins were fluorescent. He found that heating gelatin in sealed tubes increased its fluorescence. The increase was directly proportional to the amount of hydrolysis as measured by mutarotation and viscosity as well as by the Van Slyke amino nitrogen and Sorensen titration values. The gelatin was heated in sealed tubes at its isoelectric point (pH 5.05) to temperatures up to 120°C. and for times ranging up to eleven hours.

Vlès and Ugo (80) studied the fluorescence of human skin and cholesterol. They found that the human skin is much more fluorescent than cholesterol, especially when it is in the dry state. Unlike cholesterol, the skin does not give lines in its fluorescent spectrum but gives a continuous band from 2480 $\overset{o}{A}$ to the visible region.

E. Fluorescence of Insects

In 1895, F. G. Hopkins (39) published his work on the pigments of butterfly wings of the group known as <u>Pieridae</u>, which, according to him, form a large and important group of butterflies which are widely distributed. He was able to show that the white pigment in the wings was due to uric acid and that the yellow pigment was a fluorescent derivative of uric acid. This yellow pigment was characterized by the following properties: It was insoluble in ordinary organic reagents and cold water, but dissolved in hot water, from which it could be obtained as an amorphous substance. The hot solution fluoresced with a "fine green color" and the fluorescence was "well marked" at the surfaces. The substance was easily soluble in cold alkaline solutions from which it could be precipitated by neutralization. It became more fluorescent in alkaline solution when zinc chloride was added. In dilute solution it fluoresced with a blue color and gave a strong

murexide test. He claimed that a similar substance could be made from uric acid by heating a dilute sulfuric acid solution of this compound in a sealed tube to 195°C. for three hours. This synthetic yellow substance was also fluorescent.

Recently Schöpf and Kottler (73), Schöpf, Becker and Reichert (72), and Wieland and Purrmann (89) have published work on the structure of the various pterins isolated from insect wings. Although they have not yet proven the structure of any of these compounds, they have made considerable progress in characterizing them. These workers are quite agreed that only by absorption spectra data and nitrogen content are they able to distinguish one pterin from another, although there are differences in colors and fluorescence phenomena among them.

Pterins were also found to be the chief coloring matter in wasps as well as in certain species of butterflies which are yellow (71).

Dubois (11) (12) called attention to the blue-fluorescent pigments of other insects. He stated that an alcoholic extract of many insects fluoresced blue and that acetic acid caused this fluorescence to diminish; its intensity increased again when the acid was neutralized with ammonium hydroxide. He called this substance pyro-porphorin. Although he was the earliest investigator of

the fluorescence of marine animal extracts, he made no
chemical study of these materials nor of the fluorescent
material from insects which he examined.

Shortly after the publication of Dubois, Coblentz
(7) noted that the milky fluid from the body of the fire-
fly was fluorescent. In particular, the segments of the
abdomen which were luminous presented a deep blue fluores-
cence which was not lost when the material was freed from
albuminous substances. He stated that its fluorescent
band occurred between 3900 and 4900 $\overset{o}{A}$ when irradiated with
ultra-violet light.

F. Fluorescence of Plants

A very general account of the fluorescence of living
things, both plant and animal, was given by Harvey (36)
in 1926. His account contains nothing of the chemical
nature of the pigments which are present but gives the
fluorescent colors of a large number of organisms.

Suárez (77) obtained a blue-fluorescent substance
from maize which was able to sensitize blood corpuscles
and organisms to light, but when fed to animals, even in
large amounts, it did not render them light sensitive.
It has not as yet been chemically identified. It was
fat-soluble and obtained from the unsaponifiable fraction
of the fat. Suárez obtained it in crystalline form and
called it zeochin.

Metzner (55) has stated that all seeds and fruits of plants liberate fluorescent material into water when they sprout, and that these fluorescent materials are weakly photodynamic. The chemical nature of the liberated material is not known, but in general it was found that the fluorescence was more pronounced in alkaline solution and less in acid.

Nuccorini (57) found that many plants contain water-soluble blue-fluorescent substances which he claims are pigments formed as secondary products of biochemical activity of the plants. Although he gave no chemical characteristics of the material, he found that it was not injured by the action of molds.

Heilbron, Parry and Phipers (37) have shown that marine algae contain lactoflavin while fresh-water algae contain lumiflavin, the irradiation product of lactoflavin. The lumiflavin may occur in the algae in the combined or uncombined form. That occurring in the uncombined form renders algae fluorescent in the blue region of the spectrum.

Of course, it is well known that chlorophyll is fluorescent in the red and that many other plant products, such as alkaloids, are fluorescent; however, they will not be reviewed here. Andant (1) has made a careful study of the fluorescence of certain alkaloids.

Although several workers have noticed the fluorescence of various parts of many plants, no attempt has been made to isolate the causative substance or to explain its function.

G. Fluorescence of Micro-organisms and Fungi

The fluorescence of the Pseudomonas fluorescens bacteria has been known for a long time. However, it was not until 1931 that Georgia and Poe (21) (22) found the optimum conditions of culture for the production of the fluorescent pigments by these bacteria. They found that for Pseudomonas fluorescens and closely allied species it was necessary for the nutrient media to contain asparagine, phosphate, sulfate, and magnesium in order to produce the pigment. However, they did not investigate the chemical properties of the green-fluorescent material.

Pulvertaft (65) made the statement that "all bacteria grown on any medium fluoresce in ultra-violet light." Working with the more fluorescent types, he found that their pigment could be extracted from the bacterial cells with alcohol and acetone. He found the pigment to be insoluble in ether or chloroform; non-volatile, and stable to heat in N/20 hydrochloric acid but destroyed by only moderate heating in N/20 sodium hydroxide. He also found that the fluorescent color did not change with changes in pH.

When grown on solid media there appeared in the media, as well as in the bacterial cells, a fluorescent compound which could be extracted with acetone and alcohol; but in liquid media, the native fluorescence of the fresh media was enough to mask any fluorescence of the bacterial cells.

Pett (62) isolated an organism from dilute flavin solutions which was able to convert flavin into a blue-fluorescing compound which was soluble in chloroform. This substance could also be extracted from the bacterial cells as well as from the flavin media. Its fluorescence was intense in alkaline solutions (pH 12-7) but disappeared in acid solutions below pH 5. It was not affected by sodium hydrosulfite.

This same author (63) also published data to show that, under certain conditions of growth, yeast could be made to produce a blue-fluorescing substance in place of flavin which they ordinarily produce. He claimed that this blue-fluorescing substance was related to flavin but not identical with it. However, Gourévitch (28) was unable to obtain the same results. He grew yeast in the presence of cyanides, which Pett had claimed stimulated the production of the blue-fluorescing substance, but found no increased production of flavin nor was he able to find the blue-fluorescent substance described by Pett.

Cortese (9) studied the influence of anaerobiosis,

pH of the medium, and chemical constitution of the medium, on the fluorescence of _Actinomycetes_. He found that the fluorescent material could be extracted from the solid media with dilute alcohol and purified by shaking with acidified ether. It was found to be an amorphous, odorless, reddish-brown material which was soluble in alkalies, pyridine, ethyl alcohol, methyl alcohol and acidified ether. Although it was slightly soluble in acetone, it was insoluble in water, glycerine, benzene or other fat solvents. It was found to be a pigment belonging to the group of porphyrins.

Crowe (10) grew diphtheria organisms on synthetic media and then filtered them off by passing the liquid through an ultra filter. The filtrate was extracted with chloroform after removal of ether-soluble substances. The green fluorescence of the chloroform layer changed to blue when the solution was exposed to daylight. It was found that this fluorescent material was quite similar to lumiflavin but was not fully identified.

Probably the most complete study of bacterial fluorescence was made by Giral (24). He studied fluorescent bacteria grown in synthetic liquid media. In the presence of ammonia the fluorescent material has a weak green color and a blue-green fluorescence, while in stronger alkali it is more intensely green in color and more yellow-green in

fluorescence. In strong mineral acids the color is lost
and the fluorescence changes to an intensive violet color.
It is reversibly reduced by sodium hydrosulfite and is de-
stroyed by the oxidative action of the air in an alkaline
medium. It dialyzes readily and cannot be extracted by or
taken up in organic solvents. It is readily adsorbed on
charcoal and Frankonite from which it can be eluted by 30
per cent acetone and 85 per cent pyridine, respectively.
It cannot be distilled nor sublimed, but is precipitated
by phosphototungstic acid in acid solution and by barium
hydroxide from concentrated solutions. It can also be pre-
cipitated by gold chloride or picrolonic acid. It is free
from sulfur but very rich in nitrogen. Its absorption spec-
trum is similar to that of lactoflavin and xanthopterin. It
is probably intermediate between lyochromes (alloxazine de-
rivatives) and pterins (purine derivatives), since it has
properties which are intermediate between these two types
of compounds. The bacterial pigment differs from the
flavins in being destroyed by hydrogen peroxide, bromine,
and nitric acid which do not destroy flavins. It is similar
to flavins in that it gives a negative murexide test, is
reversibly reduced with hydrosulfite, is adsorbed from acid
solutions onto acid silicates, is eluted from these with
pyridine, and has a similar absorption spectrum. It re-
sembles xanthopterin in that its fluorescence varies with

changes in pH, its absorption spectrum is like that of xanthopterin, and it is adsorbed from acid solution by silicates; it is eluted from these adsorbants with pyridine, is sensitive to oxidation agents, is precipitated by barium hydroxide or gold chloride, and is reversibly reduced by hydrosulfite. It differs from xanthopterin in that it has a negative murexide test, is more soluble in water than xanthopterin, and is unstable to air in alkaline solution.

Wager (81) was the first to give a general account of the fluorescent pigments in the fungi. He stated that the fluorescence was only visible in solutions of the coloring matter extracted from the fungi by solvents. He gave a list of the fungi which give fluorescent pigments.

Josserand and Netien (43), using light of wavelength 3660 Å, examined 175 species of fungi for differences of fluorescence in many of their tissues. They state the fluorescent colors are generally some shade of yellow, blue, or violet. They made no detailed chemical study of the fluorescence. The fluorescent material is not extracted by organic solvents.

III. EXPERIMENTAL

A. Source and Treatment of Materials

1. Casein.

Commercial casein, obtained from Wilkens-Anderson Company of Chicago, was purified by washing at its iso-electric point with a dilute solution of acetic acid. The washing was continued for two weeks by daily decantation, then washed well with distilled water for two days. The product was then dried at 85°C. This protein was used in those experiments where washed casein is specified.

In those experiments where purified casein is specified, a laboratory casein preparation was used. It was prepared according to the directions of Clarke (6) as follows: To one liter of skim milk 0.05 M hydrochloric acid is slowly added with stirring through a capillary tube extending to the bottom of the beaker. The addition is continued until the solution attains a pH of 4.6. Approximately 1000 cc. of acid is required; the separation of casein is practically complete at this point. Three liters of water is then added, stirring is discontinued, and the flocculent precipitate of casein is allowed to

settle in the refrigerator for twelve to twenty-four hours.
The clear supernatant liquid which contains soluble pro-
teins and salts is removed as completely as possible by
siphoning; the precipitate is collected on a suction fun-
nel and washed with cold water until the washings are free
from calcium.

The casein, which is contaminated with salts and fats,
is filtered off to as small a volume as possible (about
500 cc.) and transferred to a 2-liter beaker. It is then
treated with 0.1 M solution of sodium hydroxide, the alkali
being added slowly and with stirring through a capillary
extending to the bottom of the beaker. The addition of
alkali is continued until the pH of the mixture reaches
6.3; 100-150 cc. of the alkali is required. At this pH
the casein is completely soluble in the form of its sodium
salt; fats, calcium phosphate and any calcium caseinate
remain undissolved. Care must be taken not to add more
alkali than is necessary to bring the pH to the above
point. The milky solution is filtered through a thick
layer of filter paper pulp tightly packed upon a suction
funnel. The filtrate is brought to a pH of 4.6 with
0.05 M hydrochloric acid just as in the original precipi-
tation, the necessary amount of acid being determined by
titration of an aliquot portion, diluted five-fold, with
0.01 M hydrochloric acid; 220-250 cc. of 0.05 M acid is

required. As the precipitation progresses, the rate at which the acid is added is decreased in order to prevent precipitation at the tip of the capillary tube; vigorous mechanical stirring is, of course, necessary. When the acidification is complete, five liters of cold distilled water is added and the flocculent precipitate allowed to settle in the refrigerator. After siphoning off the supernatant liquid, the casein is collected on a suction funnel, using hardened paper, washed with cold distilled water until free of chloride, sucked dry as possible and dried over calcium chloride in a vacuum desiccator. The yield is 23-29 g. of a colorless coherent product which may be readily pulverized in a mortar.

2. Gelatin.

This was purchased from the Difco Laboratories, Incorporated, Detroit, Michigan. It was "Difco" standardized grade which gave a negative Hopkins-Cole test for tryptophane and a very weakly positive Millon test for tyrosine.

3. Zein.

This protein was prepared from yellow corn according to the directions of Osborne and Clapp (59) modified as follows: Ground corn was extracted with 85 per cent (by volume) alcohol as long as anything was removed. The

voluminous extract was filtered perfectly clear and concentrated under reduced pressure to a small volume. The clear syrup that remained, after cooling, was poured in a very fine stream into about eight volumes of distilled ice water containing a very little sodium chloride in order to promote the separation of the zein. The zein, which separated as a flocculent precipitate, soon settled as a coherent plastic mass. This was filtered out on cloth, washed superficially with water, and again dissolved in 95 per cent alcohol and evaporated to a syrup under reduced pressure. This solution was then extracted with petroleum ether several times to remove the color and fat.

The perfectly water-clear solution of zein was then poured into eight volumes of cold water, containing a little sodium chloride. The zein that separated was then filtered off, dried by washing with ether-alcohol mixture and finally pure ether. The zein was entirely colorless but contained a slight amount of sodium chloride.

4. Gliadin.

This preparation was made from wheat flour according to the directions given by Morrow and Sandstrom (56) as follows: Place 350 g. of wheat flour in a suitable container and, while stirring, gradually add distilled water until a stiff dough is formed. Knead the dough thoroughly

and allow it to remain under water for at least half an
hour. Then wash out the starch by kneading it in a stream
of water. Cut or tear this moist gluten into very small
pieces and place in a flask; add sufficient 95 per cent
ethyl alcohol and distilled water to form, with the water
in the gluten (about 60 per cent), 600 cc. of 70 per cent
ethyl alcohol by volume. Heat the flask in a water bath
at 70°C. under a reflux condenser for an hour and a half,
shaking at frequent intervals. Filter and concentrate the
clear filtrate to a thick syrup in a 1000-cc. Claisen
distilling flask under diminished pressure until it is
impossible to keep the froth from going over into the
distillate. Use the distillate for a second and third
extraction of the gluten in the manner just described.
Glutenin was prepared from the gluten residue. Cool the
syrup in the Claisen flask, and then pour it slowly, with
constant stirring, into a large volume of ice-cold water,
containing 10 g. sodium chloride per liter; the gliadin
separates as a gummy mass. Wash it several times with
distilled water, then filter. Dissolve in 70 per cent
ethyl alcohol, concentrate the clear alcoholic filtrate
to a syrup under diminished pressure, reprecipitate by
pouring into distilled water containing 1 per cent of
sodium chloride, redissolve in 70 per cent ethyl alcohol,
and filter. Finally pour the syrup into a mixture of

absolute ethyl alcohol and ether. The gliadin separates
in flocculent form. Filter the gliadin and wash it with
absolute ethyl alcohol and finally with ether. This
gliadin contains a small amount of sodium chloride.

5. Glutenin.

The gluten residue from the isolation of gliadin was
used in the preparation of glutenin. Dissolve the gluten
residue in 4 cc. of 0.15 per cent sodium hydroxide for
each gram of gluten; after a short time filter off any
insoluble material. Neutralize the filtrate with a very
dilute solution of hydrochloric acid; the glutenin separates
in the flocculent form. Allow it to settle, decant the
supernatant liquid; wash several times by decantation
using distilled water, then several times by decantation
using 70 per cent ethyl alcohol, and last with 95 per cent
ethyl alcohol. Finally dehydrate the glutenin with abso-
lute alcohol, wash with ether and dry in a desiccator.

6. Gluten.

The gluten used in the following experiments was
isolated from wheat flour as directed above in the prepa-
ration of gliadin.

7. Blood Fibrin.

This protein was a crude commercial product and was

"purified" by dissolving in 2 per cent sodium hydroxide solution and filtering off the insoluble material. The filtrate was neutralized with 2 per cent hydrochloric acid to maximum precipitation and filtered on a pad of macerated filter paper, washed with distilled water and dried at 85°C.

8. Amino acids.

The amino acids used in these experiments, with the exception of tyrosine and cystine which were prepared in the laboratory, were purchased from Eastman Kodak Company, Rochester, New York. They were not recrystallized.

9. Fuller's earth.

Cenco fuller's earth was obtained from the Central Scientific Company, Chicago, Illinois.

English fuller's earth was purchased from the Eimer and Amend Company of New York, N. Y.

Before being used as adsorbants these earth products were first extracted with normal hydrochloric acid by mechanically stirring the powdered earth into the acid and filtering off the undissolved portion. The residue was washed with distilled water by decantation several times and then filtered on a Buchner funnel. This material was then stirred into 50 per cent methyl alcohol mixed with concentrated ammonium hydroxide in the proportion of 80

parts of the alcohol to 20 parts of ammonium hydroxide,
and filtered. The insoluble earth was washed several times
with distilled water and dried. It was found that the
above washing process was necessary to remove blue-fluorescent
material from these products.

10. Norite.

Norite "A" (decolorizing carbon) was purchased from
the Pfanstiehl Chemical Company, Waukegan, Illinois. It
was also washed before use as described under fuller's
earth above.

11. Hydralo.

This activated alumina was obtained from J. T. Baker
Chemical Company, Phillipsburg, New Jersey. Before being
used as an adsorbant it was washed with dilute ammonium
hydroxide and methyl alcohol, dried and ground in a mortar
until it passed a 40-mesh screen.

12. Hyflo Super Cel.

Hyflo Super Cel F. A. 501 (a heat treated siliceous
earth) is manufactured by Johns-Manville Company, New York,
N. Y. This material was mixed with Hydralo to speed up
the chromatographic adsorption and aid filtration.

13. **Crystalline vitamin B_1 hydrochloride.**

Synthetic crystalline vitamin B_1 (thiamin) hydrochloride was obtained from Merck and Company of Rahway, New Jersey.

14. **Flavine.**

Labco Lactoflavin--PX grade was purchased from the Borden Company, Research Division, Bainbridge, New York. It was guaranteed to be pure crystalline lactoflavin; it was purchased in the dry crystalline form.

B. Apparatus

1. **Ultra-violet light source.**

Routine examinations for fluorescence, as well as quantitative determinations, were carried out in the ultra-violet light from an Eveready Fluoray lamp. This lamp was purchased from the National Carbon Company, Cleveland, Ohio. It is a portable carbon arc burning C carbon electrodes at 30 volts and 9.0 amperes. The emitted light passes through a moulded 4.0 mm. thick, heat-resisting red-purple filter No. 587 which transmits only radiations between about 3100 and 4100 Å and very slightly in the red (about 7200 Å).

For spectroscopic work, where long exposures were necessary, a quartz mercury-vapor arc provided the source

of ultra-violet for the excitation of the fluorescence.
Light from this mercury arc was passed through the filter
mentioned above before illuminating the solutions whose
fluorescence was being photographed.

2. Spectrometer.

A Gaertner wavelength spectrometer which had the
telescope replaced by a spectroscopic camera attachment
was used for photographing the fluorescent spectra. This
instrument is a constant deviation instrument, the prism
being of the Pellin-Broca type.

3. Photographic plates.

The plates used were Wratten and Wainwright panchro-
matic dry plates made by Eastman Kodak Company. These
plates were developed in Eastman formula D-19.

C. Method of Protein Hydrolysis

During the early orientation experiments it was found
that reagents and materials must not be allowed to come in
contact with any rubber or cork. When allowed to contact
these materials, especially at elevated temperatures, the
reagents remove from the rubber and cork a considerable
amount of blue-fluorescent material which greatly inter-
feres with the quantitative determination of the amount of

fluorescence of the proteins. Therefore, all reagents were examined for fluorescence just prior to their use and only non-fluorescent reagents were selected. Solid reagents were dissolved in distilled water and examined just before use. When it became necessary to purify liquid reagents, simple redistillation was generally sufficient to remove all fluorescence.

Blank determinations on the reagents used must be run on each experiment to insure against fluorescence appearing from chemical reactions. However, when non-fluorescent reagents are selected the blank determinations show no appreciable fluorescence.

Proteins were hydrolyzed in Florence flasks equipped with closely fitting test tube condensers inserted into the necks of the flasks. The flasks were heated over air baths and during the first part of the digestion were often shaken to prevent any heat decomposition of the undissolved protein. Before a sample is taken for fluorescence determination, the volume is made to its initial volume with distilled water when non-volatile acid or base was used for the hydrolysis. When volatile acids were used, the initial volume was restored with the acid. Care was taken to prevent loss and changes in concentration of the hydrolysis agents.

D. Fluoremetric Method

Radley and Grant (66) and Haitinger (33) have reviewed the methods of analysis by fluorescence which have been used. The most modern, and probably the most accurate, methods involve the use of a nephelometer fitted with a step photometer for measuring the intensity of the emitted fluorescent light. However, the intensity of the fluorescence is not directly proportional to the concentration of the fluorescent material for known substances and therefore the instrument must be calibrated with known amounts of the material under investigation. Therefore, when unknown material is analyzed by these instruments serious difficulties are encountered. Other factors such as pH, color, and quenching substances present, must be taken into account and the proper corrections made when possible.

A fairly accurate method which is simple and inexpensive has been developed by the author for use in determining the relative amounts of fluorescence in proteins. Gerngross and Hübner (23) used simple dilution to determine the amount of fluorescence in unknown materials. It is a well known fact that as the concentration of the known fluorescent materials approaches zero, the fluorescence also approaches zero. Over the low concentration ranges this relationship between concentration and fluorescence

is a straight line function. By comparing fluorescent intensities which are very weak, and using very dilute solutions, it is possible to make quite accurate comparisons of unknown concentrations on this basis. When known fluorescent compounds are used, the concentrations can be determined within 5 per cent of the calculated values in the case of quinine bisulfate, when this method of dilution and comparison with known standards of low concentration and fluorescence is used.

The method used to determine the amount of fluorescence in the experiments reported in this thesis is as follows: A small accurately measured volume (usually one cc.) of unknown hydrolysate or protein solution is diluted to 100 or 200 cc. depending on the strength of its fluorescence. This solution is placed in a burette and titrated into 175 cc. of dilute acid (0.1 $\underline{N}$ HCl) until the intensity of the solution in the 300-cc. Erlenmeyer flask just matches that of the standard quinine bisulfate solution. This standard quinine solution is prepared as follows: Weigh out 0.0286 g. of quinine bisulfate (Merck) and dissolve in 0.5 $\underline{N}$ sulfuric acid. Make to one liter with the dilute sulfuric acid. After mixing, dilute 1.0 cc. of this solution to 200 cc. with 0.5 $\underline{N}$ sulfuric acid. This standard will keep for at least a week and is easily duplicated by diluting the stronger quinine

solution. The standard is kept in a 300-cc. Erlenmeyer flask closed by a stopper wrapped in tin foil. (The solution must not be allowed to contact cork or rubber.)

Comparisons are made in 300-cc. pyrex Erlenmeyer flasks which have been previously examined for fluorescence. Only non-fluorescent flasks are used. All comparisons are made in the light from an Eveready Fluoray lamp in a dark room. The final dilution of the unknown must be near the same volume as the standard (200 cc.) in order to get consistent comparisons. All flasks are placed on a non-fluorescent black table top during comparisons with the standard.

The pH of the solution used for comparison affects the results; if the solution becomes alkaline, the results are always lower than when kept acid. Therefore, all solutions must be kept acid during the comparisons. A small excess of acid does not change the results, but strongly acidic solutions should be avoided.

The color of the fluorescence from protein hydrolysis is very nearly the same as that of the quinine bisulfate and with very little practice duplicate titration values can be readily obtained.

Although great accuracy is not claimed for this method of determining the relative concentrations of fluorescent material in proteins, it must be pointed out that the values can be readily duplicated. Burette readings can be

easily duplicated within 1.0 cc. when about 20 cc. are used
in the titrations. This amounts to a variation of 5 per
cent in the fluorescent values as reported.

The results are expressed as the dilution (in liters)
per gram of original protein required to produce the same
fluorescence as the standard solution of quinine bisulfate.

E. Presentation of Data

1. Fluorescence of solid proteins.

When solid proteins are examined in ultra-violet light,
they exhibit a bluish-white fluorescence which appears to
be the same for all proteins both as to color and intensity.
If concentrated protein solutions are evaporated on glass
slides, the fluorescence appears to be the same regardless
of the acidity of the solution from which they were de-
posited. Thus, gelatin has the same colored fluorescence
whether the solid be deposited from neutral, acidic or
basic solution provided that hydrolysis is prevented during
the evaporation. That the fluorescence is closely associ-
ated with the protein and not due to adsorbed or occluded
material is shown by the fact that dialysis of protein sus-
pensions against distilled water for long periods of time
did not remove an appreciable amount of fluorescent material
nor did it reduce the amount of fluorescent material formed

when these proteins were hydrolyzed. Also, when ashed, the small amount of protein ash is neither fluorescent in the solid state nor when dissolved in water, dilute acid or base.

When proteins are dissolved in strong nitric acid, destruction takes place and the resulting solution is not fluorescent in the visible region. When nitrates are added to acid-hydrolyzed proteins there is no quenching or interference with the amount of fluorescence, indicating that the nitric acid causes destruction of the fluorescent material of the proteins.

2. Fluorescence of protein solutions.

The fluorescence of protein solutions is not intense. In solution, various proteins exhibit a fluorescence which is somewhat more green than the fluorescence of the solid protein. Protein solutions also show strong absorption of the ultra-violet light so that only thin layers of solution next to the incident light appear fluorescent. However, in dilute solutions the fluorescence appears throughout the entire solution and is a uniform bluish-green for all proteins examined. Acid meta-protein solutions fluoresce with the same color as alkaline meta-protein solutions. This soluble fluorescent material is not removed by dialysis of the protein solution in collodion bags

suspended in running distilled water. Neither is it removed by dialysis from slightly acid or alkaline protein solutions.

3. Fluorescence of hydrolyzed proteins.

Hydrolysis by boiling acids or alkalies and the action of proteolytic enzymes have yielded much of the present available information on the structure and composition of the proteins. Since Pringsheim and Gerngross (64) found that the hydrolysis of gelatin increased its fluorescence, other methods of hydrolysis of various proteins were studied in an attempt to determine the cause and nature of the fluorescence.

It was found that when proteins are hydrolyzed with acids, the amount of fluorescence, as measured by the dilution required to reduce its intensity to a small, known value, was greatly increased.

a. Hydrolysis by hydrochloric acid. When proteins are hydrolyzed by concentrated hydrochloric acid the fluorescence of the resulting solution is somewhat masked by the large amount of black insoluble humin material formed during the hydrolysis. However, when this material is removed by filtration, or when the solution is diluted with distilled water until it is colorless, the fluorescence is readily observed in ultra-violet light. The fluorescence of the

filtrates from the removal of the insoluble humin is strong enough to be observed in diffuse daylight. This fluorescence always appears greenish-blue in acid solution and somewhat more green and not so intense in basic solution.

During the preliminary experiments, it was found that various proteins gave the same fluorescent color when hydrolyzed and that all appeared intensely fluorescent. However, when the amounts of fluorescence obtained during hydrolysis of these proteins were compared, marked variations were discovered. It was found that blood fibrin produced about 30 times as much fluorescence as a similar amount of gelatin or zein when hydrolyzed under the same experimental conditions. One gram of blood fibrin when hydrolyzed with concentrated hydrochloric acid for 36 hours requires a dilution of 60 liters to reduce the intensity of its fluorescence to that of the standard quinine bisulfate solution used; one gram of gelatin or zein, when hydrolyzed in a similar manner, requires a dilution of only two liters to produce the same intensity of fluorescence.[1] Other proteins have fluorescence values intermediate between gelatin and blood fibrin.

Table I shows the fluorescence values of various proteins after hydrolysis for various periods of time with concentrated hydrochloric acid. These values are expressed as the volume, measured in liters, to which one gram of the

1. The fluorescence color is always greenish-blue unless otherwise stated.

Table I. Fluorescence values of proteins hydrolyzed
with concentrated hydrochloric acid.

Kind of protein	c.c. of protein	HCl	Fluorescence values after hydrolysis for:					
			0 hr.	2 hr.	6 hr.	12 hr.	24 hr.	36 hr.
Casein (washed)	10	100	0.6	10	17	22	21	22
Egg albumin (commercial)	5	100	0.7	—	24	42	41	42
Fibrin (blood)	5	100	—	—	54	64	58	60
Gelatin (Bacto)	10	100	0.2	2	2	2	2	2
Gliadin	10	100	—	—	53	50	46	51
Glutenin	10	100	—	40	56	57	54	—
Gluten (wheat)	10	100	—	—	35	46	45	44
*Hair (human)	10	100	—	—	21	29	28	32
*Wool	10	100	—	—	31	31	30	28
Zein	5	100	0.2	2	2	2	2	2

*Wool and hair were thoroughly cleansed and extracted with alcohol and ether for several hours before hydrolysis.

protein must be diluted in order to produce the weak fluorescence comparable in intensity to the standard quinine bisulfate solution (0.143 mg. per liter) to which all comparisons were made as described above.

The values given in Table I are averages of two or more closely agreeing determinations. These data show that the fluorescence of proteins is greatly increased by acid hydrolysis and that blood fibrin is much more fluorescent than other proteins after hydrolysis. Zein and gelatin upon hydrolysis with acid produce an increased amount of fluorescence but the increase is much less than that of other proteins.

The material present in the hydrolysates which is responsible for the fluorescence can be dialyzed through collodion or parchment membranes. It can be concentrated as will be shown later. Since the hydrolysates do not give a positive biuret test after long boiling, the fluorescent material produced is probably not a polypeptid. Under the conditions of hydrolysis it is not likely that peptids would remain unhydrolyzed to account for the increased fluorescence.

b. Hydrolysis with other strong acids. A comparison of the fluorescence values of casein when hydrolyzed with various strong acids shows that hydrochloric, perchloric, phosphoric, and sulfuric acid causes increased fluorescence

of casein during hydrolysis and that nitric acid destroys the fluorescence. That the action of the nitric acid is destructive and not merely quenching is shown by the fact that fluorescent hydrolysates when mixed with cold nitric acid are fluorescent, but if heated in the presence of this acid, the fluorescence is entirely destroyed. Moreover, the addition of nitrates to fluorescent hydrolysates does not inhibit their fluorescence showing that the nitrate ion does not cause quenching.

Table II shows the fluorescence values of casein when hydrolyzed by various strong acids of the same normality (12 $\underline{N}$). The fluorescence values are expressed as previously described.

The explanation for the increased fluorescence values when perchloric, phosphoric, and sulfuric acids are used as compared to hydrochloric acid, is not clear. However, the data show that the increased fluorescence value of casein becomes constant after six hours hydrolysis in hydrochloric or perchloric acids. When sulfuric or phosphoric acid is used in the hydrolysis, the fluorescence value of casein becomes constant only after 24 hours or more. These hydrolysis periods, at which constant fluorescence values are obtained, correspond roughly to the time required to produce complete casein hydrolysis as measured by the biuret test. According to Schmidt (70)

Table II. Fluorescence values of casein after
hydrolysis with various 12 N acids.

Kind of acid	g. casein	cc. 12 N acid	Fluorescence values after hydrolysis for:					
			2 hr.	6 hr.	12 hr.	24 hr.	36 hr.	48 hr.
Hydrochloric	10	100	18	21	23	21	20	24
Nitric	10	100	0	0	0	0	0	0
Perchloric	10	100	31	46	41	49	41	41
Phosphoric	10	100	9	21	34	58	60	56
Sulfuric	10	100	28	37	37	43	56	56

hydrochloric acid produces hydrolysis of proteins as rapidly as sulfuric acid of the same normality. The relative rates of hydrolysis of proteins in the presence of the other acids have not been studied. From the above data obtained on casein, it appears that the maximum fluorescence values are obtained only when complete protein hydrolysis has occurred.

In order to make certain that the fluorescence values of the protein hydrolysates are not dependent on the degree of purity or the previous treatment of the protein, various samples of the same protein were compared during acid hydrolysis. The results are given in Table III. The washed casein refers to casein which was washed at its isoelectric point for two weeks. The purified casein was prepared according to the directions of Clarke (6) and had been re-precipitated three times from dilute sodium hydroxide solution by the addition of dilute hydrochloric acid, and washed with distilled water after each precipitation. The crude gelatin was a commercial product containing a considerable amount of calcium sulfate and was quite brown in color. Zein preparation I was prepared according to the directions of Osborne and Clapp (59) while preparation II was of undetermined origin but probably of good quality as indicated by its white color and negative Hopkins-Cole test.

Table III. Fluorescence values of treated and untreated
proteins during acid hydrolysis.

Kind of protein	Treatment	g. protein	cc. of HCl	conc. of HCl %	Fluorescence values after hydrolysis for: 6 hr.	14 hr.	28 hr.	36 hr.
Casein (crude)	none	10	100	25	22	22	20	19
Casein	washed	10	100	25	16	24	24	22
Casein	purified	10	100	20	20	18	22	22
Casein	washed	10	100	20	16	18	26	24
Gelatin (Bacto)	none	10	200	25	1	1	1	—
Gelatin (crude)	none	10	200	25	2	1	1	—
Zein	Prep. I	2	100	36	2	2	2	2
Zein	Prep. II	2	100	36	2	2	2	2

The results indicate that further purification of the protein preparations does not influence the amount of fluorescence obtained on acid hydrolysis.

Wels and Jokisch (86) found that the fluorescence of irradiated proteins was increased in the presence of oxygen and was always less if irradiated in the absence of oxygen. In order to determine the influence of oxygen on the production of fluorescence during acid hydrolysis, air was excluded by carrying out the hydrolysis in an atmosphere of purified nitrogen. Table IV gives the fluorescence values in the presence and absence of air during hydrolysis in concentrated hydrochloric acid.

These data show that the exclusion of oxygen from the hydrolysis does not influence the amount of fluorescence of casein hydrolysates, and that the fluorescent material produced is stable to long periods of boiling in strong hydrochloric acid.

Although accurate data were not obtained, it was found that the addition of hydrogen peroxide to the hydrolyzing proteins did not appreciably change the amount of fluorescence produced, nor did the addition of small amounts of metallic zinc during hydrolysis seem to influence the fluorescence.

c. Hydrolysis with alkali. It is generally agreed that strong alkalies produce rapid and complete hydrolysis

Table IV. Fluorescence values during acid hydrolysis of casein in the presence and absence of air.

Protein	g. used	cc. conc. HCl	Fluorescence values after hydrolysis for:							
			4 hr.	12 hr.	18 hr.	24 hr.	36 hr.	48 hr.	60 hr.	70 hr.
Casein (N_2)	30	200	15	22	22	22	28	22	28	28
Casein (air)	30	200	11	16	21	21	22	21	26	26

of the proteins. However, during alkaline hydrolysis there is complete racemisation of the amino acids formed, as well as destruction of cystine and arginine. Acid hydrolysis produces only slight racemisation of the amino acids but destroys all of the tryptophane. Alkaline hydrolysis, on the other hand, does not destroy tryptophane.

Table V gives a summary of the data obtained by the hydrolysis of proteins with alkalies. The biuret test was applied to determine when complete hydrolysis had taken place and the Hopkins-Cole test was applied to show that tryptophane was not completely destroyed.

The Hopkins-Cole test was positive on all hydrolysates, except gelatin, even after 36 hours hydrolysis, showing that all of the tryptophane was not destroyed. Gelatin does not give a positive Hopkins-Cole test. The biuret test became negative very early in the hydrolysis of gelatin and blood fibrin, especially with the stronger alkali. Casein and gluten are more resistant to alkaline hydrolysis than gelatin or fibrin; with the weaker alkaline solutions complete hydrolysis of casein was not obtained even after 36 hours boiling.

These fluorescence values obtained during alkaline hydrolysis are not as high as those obtained during acid hydrolysis of the same proteins. Gelatin gives very low fluorescence values when either acid or alkali is used for

Table V. Fluorescence values of proteins during alkaline hydrolysis.

Protein	g. protein	cc. of alkali	Kind of alkali	conc. of alkali %	Fluorescence values after hydrolysis for:				
					2 hr.	6 hr.	12 hr.	24 hr.	36 hr.
Casein	10	100	NaOH	20	1	8	8	7*	12*
Casein	10	100	KOH	10	1	8	8	6	7
Casein	5	100	NaOH	0.5	2	4	4	8	-
Casein	10	100	Ba(OH)$_2$	10	-	8	7	8	8
Fibrin (blood)	10	100	NaOH	20	2*	8*	8*	11*	11*
Fibrin "	10	100	KOH	10	2	8	8	11	10*
Gelatin	10	100	NaOH	20	1*	2*	2*	2*	2*
Gelatin	10	100	KOH	10	1	2*	2*	2*	2*
Gluten (wheat)	10	100	NaOH	20	3	8	8*	17*	17*
Gluten (wheat)	10	100	KOH	10	3	8	8	10	9*

*Biuret test negative.

its hydrolysis. Other proteins give less fluorescence when hydrolyzed with alkali than with strong acids.

d. Hydrolysis with enzymes. When proteins are hydrolyzed by enzymes, the fluorescence is not greatly increased above the fluorescence of the undigested protein provided that bacterial action is inhibited. When there is extensive bacterial action during enzyme hydrolysis the fluorescence values are higher than when bacterial action is inhibited. Table VI gives the fluorescence values of proteins during hydrolysis by trypsin (pancreatin). In these experiments, weighed portions of protein were placed in 250-cc. Erlenmeyer flasks with 100 cc. of distilled water. Five-tenths gram of pancreatin was added and the pH adjusted to 8.3 with solid sodium carbonate.[1] A small amount of chloroform was added along with enough toluene to form a layer over the mixture to prevent oxidation and bacterial action. The flasks were incubated at 40-43°C.

It is believed that some of the increased fluorescence in these hydrolyses was due in part to bacterial action, since when sodium fluoride was added to the digestion mixtures in other experiments, the fluorescence values were less than when no fluoride was added. Thus, in an experiment using casein, when 3.0 grams of sodium fluoride was added to 250 cc. of digestion mixture containing 50 grams of casein during an incubation of three weeks, the

1. The pH is 8.3 when sodium carbonate was added until the solution gives a strong pink color with phenolphthalein.

Table VI. Fluorescence values during hydrolysis with trypsin (pancreatin).

Kind of protein	g. used	Fluorescence values after hydrolysis for:							
		12 hr.	36 hr.	60 hr.	90 hr.	118 hr.	162 hr.	204 hr.	252 hr.
Casein	10	2	4	2	4	4	4	8	11
Egg albumin	10	1	1	1	1	1	1	3	3
Fibrin (blood)	10	1	3	4	4	6	6	8	8
Gluten (wheat)	10	8	6	9	8	9	6	8	10
Gliadin	10	5	8	9	11	11	8	10	12
Glutenin	10	1	3	4	4	4	2	13	11
Gelatin	10	1	1	1	2	4	4	6	8
Zein	5	1	3	3	3	5	5	10	11
Blank	0	0	0	0	0	0	0	slight	slight

fluorescence value was only 2; in the control flask with no added sodium fluoride, the fluorescence value was 13. Likewise, when no toluene was added, the values were variable and always higher than when bacterial action and oxidation were controlled. That the sodium fluoride does not inhibit the fluorescence is shown by the fact that when added to protein hydrolysates it does not change the fluorescence intensity or color.

The results of experiments using protein digestion with pepsin followed by trypsin digestion are given in Table VII. In these experiments, 10 grams of protein, 200 cc. of distilled water, and 1 gram of pepsin (U.S.P.) were mixed and enough dilute hydrochloric acid added to produce free acidity as indicated by Topfer's reagent. The flasks were incubated at 40-43°C. and kept acid by the daily addition of the required amount of dilute hydrochloric acid to produce free acidity. After the pepsin digestion had become constant, as measured by the Sorensen titration method, 1 gram of pancreatin was added and the pH adjusted to 8.3 with sodium hydroxide and the incubation continued. Further digestion occurred when pancreatin was added, as shown by the Sorensen titration method of measuring the amino nitrogen content of the digestion mixture. The blank showed very slight fluorescence of the enzymes.

Table VII. Fluorescence values of proteins during hydrolyate
with pepsin followed by trypsin (pancreatin).

Protein used	G. used	pepsin hydrolyate for: Fluorescence values during				trypsin hydrolyate for: Fluorescence values during			
		6 hr.	24 hr.	72 hr.	132 hr.	16 hr.	52 hr.	112 hr.	200 hr.
Casein	10	1	1	1	1	1	2	2	4
Fibrin	10	1	1	1	1	1	2	2	4
Gelatin	10	1	1	1	1	1	2	2	4
Gluten	10	2	2	2	4	2	4	4	8
Blank	--	0	0	trace	trace	trace	trace	trace	trace

The results of enzyme hydrolysis of proteins indicate that there is only a slight increase in the fluorescence values of these proteins during enzymatic hydrolysis provided that bacterial and oxidative enzymes are inhibited.

The fact that hydrolysis had taken place during the enzyme digestion is shown by the increased amino nitrogen as determined by the Sorensen titration method. The data are given in Table VIII.

The values in Table VIII are given as milligrams of amino nitrogen per gram of original protein.

e. "Hydrolysis" with acetic acid. Weak acids have not been generally used for hydrolysis of proteins. Zelinsky and Ssadikow (90) used dilute mineral acids as well as acetic acid and claimed that at high temperatures complete hydrolysis of proteins could be obtained without the formation of black insoluble humin when acetic acid was used. According to these authors the time required for complete hydrolysis with acetic acid is about six hours at a temperature of 180°C.

In attempting to hydrolyze proteins with acetic acid, it was found that autoclaving casein with glacial acetic acid for eight hours at 15 lbs. pressure did not bring about appreciable hydrolysis; a gelatinous residue was left after such treatment and the solution gave a positive biuret test. In other experiments, proteins were boiled with

Table VIII. Amino nitrogen of the proteins during enzyme
hydrolysis with pepsin and trypsin (pancreatin).

Protein	g. used	Amino nitrogen content after enzyme hydrolysis for:							
		6 hr.	24 hr.	72 hr.	132 hr.	*148 hr.	184 hr.	244 hr.	328 hr.
Casein	10	2.8	5.2	8.1	8.1	17.9	25.2	22.4	25.5
Fibrin	10	2.5	4.5	8.1	9.1	15.7	18.2	20.4	19.0
Gelatin	10	3.9	4.3	4.8	5.0	10.8	14.3	14.3	16.9
Gluten	10	2.2	2.0	2.8	4.2	12.9	15.5	16.5	19.0

*Trypsin (pancreatin) was added at this point after 132 hours.

glacial acetic acid for as long as 72 hours without producing complete hydrolysis.

That the fluorescence of the proteins is greatly increased during boiling with acetic acid is shown by the data in Table IX.

When acetic acid is boiled in the absence of amino acids or protein it does not become fluorescent. When added to fluorescent hydrolysates obtained by strong acid hydrolysis, acetic acid does not affect the color of fluorescence nor its intensity. The data of Table IX indicate that the fluorescence of proteins is greatly increased in the presence of boiling acetic acid, but constant values were not obtained when boiled for 36 hours. Other experiments, using acetic acid and boiling for as long as 72 hours did not produce constant fluorescence values, nor complete hydrolysis.

Although quantitative data are lacking, wool and hair also give high fluorescence values when boiled in acetic acid although only a small amount of the protein material dissolves. In addition, the color of the fluorescence is much more blue than when mineral acids are used in the hydrolysis. There are no characteristic fluorescence values for the various proteins when treated with acetic acid as is the case with strong acid hydrolysis. When proteins are boiled with mixtures of acetic and hydrochloric

Table IX. Fluorescence values of proteins during "hydrolysis" with acetic acid.

Kind of protein	g. protein used	cc. of acid	conc. of acid %	Fluorescence values after "hydrolysis" for:				
				2 hr.	6 hr.	12 hr.	24 hr.	36 hr.
Casein (washed)	5	100	5	2	3	10	12	32
Casein "	10	100	72	20	37	33	44	52
Casein "	5	100	98	18	26	26	58	62
Egg albumin (comm.)	5	100	98	20	31	37	53	62
Fibrin (blood)	5	100	98	2	17	30	57	—
Fibrin (blood)	50	300	98	—	—	19	19	153
Gelatin	5	100	98	14	26	23	33	39
Gelatin	50	200	98	—	—	25	102	51
Gliadin	5	100	98	22	44	42	54	61
Gluten (wheat)	5	100	98	24	28	40	64	86
Glutenin	5	100	98	8	14	24	46	—
Zein	1	50	98	5	10	10	20	27

acid, fluorescence values obtained are the same as when hydrochloric acid is used alone for the hydrolysis.

When acetic acid is boiled with amino acids, fluorescence develops in many cases. Threonine, tryptophane and phenylalanine when boiled in glacial acetic acid produce strong fluorescence of a bright blue color. When cystine, aspartic acid, arginine, proline, cystine or histidine are boiled in this medium only slight fluorescence of the same color develops. Thus, acetic acid appears to react differently with the amino acids than does strong mineral acid which produces fluorescence only with tryptophane.

4. Fluorescence of amino acids.

The data obtained on the hydrolysis of proteins show that zein and gelatin differ from the other proteins in their production of fluorescence during hydrolysis with acids. These two proteins are the only ones which are deficient in tryptophane. Although these two proteins are deficient in other amino acids, tryptophane is absent from both. This would suggest that perhaps tryptophane or its derivatives are responsible for the fluorescence production during hydrolysis. In addition, since the tryptophane content of blood fibrin is known to be higher than most other proteins and since this protein gives more fluorescence during acid hydrolysis, the relationship of tryptophane to fluorescence production is further suggested.

Table X gives the tryptophane content of the various proteins studied in this investigation as obtained from the literature and the fluorescence values of these same proteins after complete acid hydrolysis. It is because of inadequate methods of tryptophane determination that values from the literature are used. In attempting to use the method recently published by Sullivan et al. (78), it was found that reproducible values could be obtained for the tryptophane content of casein, fibrin and egg albumin, but when applied to the other proteins the method was useless. The color development in most cases was poor and of variable shades of blue so that accurate comparisons were impossible. This method gives a tryptophane content of 5.4 per cent for fibrin, 1.9 per cent for egg albumin with a value of 2.2 per cent for casein. These amounts are in agreement with the accepted values for these proteins.

Table X

Tryptophane content and fluorescence
values of proteins after acid hydrolysis.

Protein	Per cent tryptophane	Fluorescence values after acid hydrolysis
Fibrin (blood)	5.4	60
Casein	2.2	22
Hair	1.8	32
Wool	1.8	28
Glutenin	1.7	54

Table X. (Continued)

Protein	Per cent tryptophane	Fluorescence values after acid hydrolysis
Egg albumin	1.3	42
Gliadin	1.1	51
Zein	0.2	2
Gelatin	0.0	2

The values for the tryptophane content of the proteins were those given by Schmidt (70) and Jones et al. (41) and are values based on several methods of tryptophane determination. Although the correlation of tryptophane content and fluorescence is not good, it must be remembered that tryptophane determinations are not satisfactory and that fluorescence values are not extremely accurate. Nevertheless, the high and low fluorescence values correspond to the high and low tryptophane content.

The amino acids were examined for fluorescence in the solid state as well as in acid, neutral and basic solutions in order to determine their influence on fluorescence of hydrolyzed proteins. The 19 amino acids examined are listed below.

dl-Alanine
 d-Arginine monohydrochloride
 l-Aspartic acid
 l-Cystine
  Glycine

l-Lysine picrate
dl-Methionine
dl-Phenylalanine
 l-Proline
dl-Serine

d-Glutamic acid   dl-Threonine
l-Histidine dihydrochloride   l-Tryptophane
l-Hydroxyproline   l-Tyrosine
dl-Isovaline   dl-Valine
dl-Leucine

In the solid form, none of the amino acids examined showed any visible fluorescence in ultra-violet light. Also when examined in neutral, acid or basic solution none exhibited fluorescence in the concentrations used (20 mg. per cc.). However, when boiled in 20 per cent hydrochloric acid for a short time tryptophane showed some fluorescence of the same color as that obtained from protein hydrolysis, namely, blue-green. Tryptophane was the only amino acid tested which gave fluorescence in hot hydrochloric acid solution. However, the intensity of the tryptophane fluorescence is quite weak.

A mixture of all the amino acids available boiled with hydrochloric acid shows fluorescence of the same color as that from proteins, but the intensity is very much less. When tryptophane was omitted from this amino acid mixture during boiling with hydrochloric acid, no fluorescence developed. Thus, it appears that of the 19 amino acids tested, only tryptophane is capable of producing fluorescence under the conditions of protein hydrolysis.

When each of the tested amino acids was added to gelatin during acid hydrolysis, increased fluorescence was obtained only when tryptophane was added. The effect of

tryptophane addition to gelatin, zein and casein during
acid and alkaline hydrolysis is shown by the data of
Table XI.

These data show that tryptophane addition to casein
does not change the amount of fluorescence formed during
acid or alkaline hydrolysis. On the other hand, when added
to the tryptophane deficient proteins zein or gelatin, the
fluorescence values of these proteins are greatly increased
during acid hydrolysis. During alkaline hydrolysis, added
tryptophane does not influence the amount of fluorescence.

Since tryptophane addition greatly increases the fluo-
rescence values during acid hydrolysis and since tryptophane
is destroyed under these conditions, the increased fluores-
cence of acid-hydrolyzed proteins is probably due to tryp-
tophane destruction.

Harvey and Robson (35) have shown that tryptophane can
be readily converted to a highly fluorescent alkaloid
(harman) by mild oxidation of the tryptophane-acetaldehyde
addition compound. However, when synthetic harman is added
to protein hydrolysates, it can be recovered by ether ex-
traction which does not remove the fluorescent material
formed from the proteins during hydrolysis. Also, the
fluorescent material from proteins does not have the
physiological action of the synthetic drug although the
fluorescence of the two are quite similar in color. It is

Table XI. Fluorescence values of proteins when
tryptophane is added during hydrolysis.

Protein	g. protein used	g. tryptophane added	Hydrolysis medium	Vol. of solution	Fluorescence values after hydrolysis for:				
					4 hr.	8 hr.	12 hr.	24 hr.	36 hr.
Gelatin	10	0	HCl (25%)	100	1	1	-	2	2
Gelatin	10	0.02	HCl (25%)	100	6	-	5	5	-
Gelatin	10	0	HCl (25%)	200	2	2	2	2	2
Gelatin	10	0.1	HCl (25%)	200	2	8	10	10	12
Gelatin	10	0.2	HCl (25%)	100	4	18	12	12	-
Gelatin	10	0.4	HCl (25%)	100	12	24	20	16	-
Gelatin	10	0.05	NaOH (20%)	100	2	2	2	2	-
Gelatin	10	0.1	NaOH (20%)	100	1	1	1	1	1
Zein	2	0	HCl (25%)	100	1	2	2	3	3
Zein	2	0.1	HCl (25%)	100	27	26	27	28	35
Casein	10	0.05	HCl (25%)	100	-	20	18	21	22
Casein	10	0.1	HCl (25%)	100	-	21	22	22	22
Casein	10	0.1	NaOH (20%)	100	4	10	11	-	-

concluded that this oxidation product of tryptophane cannot be responsible for the increased fluorescence of hydrolyzed proteins.

It is well known that vitamins are difficult to remove completely from the proteins and that certain vitamins decompose readily to form blue-fluorescent products. Therefore, the effect of added vitamins on the fluorescence during acid hydrolysis was tested. When both vitamin B_1 and lactoflavin are added to gelatin in amounts greater than ordinarily found in this protein, there was only a very slight increase in the fluorescence value of gelatin. Thus, when 5 milligrams of vitamin B_1 and 2 milligrams of lactoflavin were added to 5 grams of gelatin during hydrolysis in 100 cc. of 25 per cent hydrochloric acid, the fluorescence value of the gelatin was increased, from 1 when no vitamin addition was made, to a value of 3. Dried yeast gave a maximum fluorescence value of 20 during acid hydrolysis, indicating that vitamins of the B-complex do not cause greatly increased fluorescence.

Alloxan, a compound closely related to lactoflavin, is fluorescent in acid solution. Its color of fluorescence is similar to that of hydrolyzed protein. However, its fluorescence is readily quenched with mercuric chloride and it gives a positive murexide test. Fluorescent protein hydrolysates and concentrated fluorescent material from

them do not give a murexide test nor is their fluorescence quenched by mercuric chloride.

5. Humin formation and fluorescence.

Gortner et al. (25) have shown that tryptophane and an unidentified constituent of proteins react to form the black insoluble humin obtained when proteins are hydrolyzed in strong acids. These authors have not mentioned or dealt with the fluorescence of humin or of the protein hydrolysates.

The fluorescence of insoluble black humin is not readily detected, but when this material is dissolved in sodium hydroxide it exhibits a weak green fluorescence. Those protein hydrolysates which show the strongest fluorescence also are the darkest in color. In gelatin and zein hydrolysates, where the color is only light brown compared to the black color of other protein hydrolysates, the fluorescence is much weaker as has been shown above. The effect on fluorescence values of preventing humin formation, by the common method of adding stannous chloride during hydrolysis, is shown in Table XII. When stannous chloride is added in sufficient quantities to prevent humin formation, the hydrolysates have a light brown color but no insoluble material remains.

Table XII. Fluorescence values of proteins during hydrolysis
in the presence of stannous chloride.

Protein	g. protein used	g. SnCl$_2$ added	cc. HCl (conc.)	Fluorescence values of proteins after hydrolysis for:				
				2 hr.	6 hr.	12 hr.	24 hr.	36 hr.
Casein	10	0	100	—	20	21	24	24
Casein	10	6	100	—	9	20	20	24
Gluten	5	0	100	35	44	53	47	43
Gluten	5	4	100	9.8	19	15	17	15
Fibrin	5	0	100	—	45	54	53	56
Fibrin	5	4	100	27	28	42	34	36

These data indicate that the prevention of humin
formation by stannous chloride reduces the amount of fluo-
rescence formed during acid hydrolysis but does not com-
pletely destroy the material causing fluorescence, although
it prevents the formation of brownish-black melanin or
humin. Stannous chloride does not interfere with nor di-
minish the amount of fluorescence of hydrolysates, as is
shown by the fact that the addition of large amounts of
stannous chloride to the hydrolysates which are fluorescent
does not affect them. Stannous chloride boiled with hydro-
chloric acid is not fluorescent.

Gortner and Holm (25) produced humins by adding trypto-
phane to aldehydes in hydrochloric acid medium. Humin pro-
duced in this manner is only very slightly fluorescent when
the concentration of the acid is kept below 10-15 per cent.
With more concentrated acid, the solutions are much darker
and filtrates from the removal of the insoluble material
are very strongly fluorescent. If acetaldehyde is added
to gelatin during hydrolysis in concentrated hydrochloric
acid, a very great increase in the fluorescence is noted.
However, when acetaldehyde is boiled in concentrated hydro-
chloric acid, or even in 10 per cent acid, strongly fluo-
rescent solutions are obtained. The fluorescence of these
solutions is very similar in color to that from hydrolyzed
proteins and prevents the determination of protein fluores-
cence.

When glucose and tryptophane are condensed with acid, a blue-fluorescent compound results. This compound resembles the fluorescent material from proteins in its adsorption characteristics on fuller's earth, and also its change in fluorescent color when the pH is changed. Both of these compounds fluoresce bluish-green in basic solutions and greenish-blue in acid. However, the change in fluorescent color is not sharp nor easily detected. Of the eleven amino acids tested for fluorescence production with glucose in acid, tryptophane and tyrosine were the only ones which gave blue-fluorescent compounds. The other amino acids tested were histidine, glutamic acid, threonine, phenylalanine, cystine, aspartic acid, proline, glycine and arginine. Six-tenths millimol of each of these amino acids was added to 5 grams of glucose in 50 cc. of hydrochloric acid and boiled for six hours. All of the solutions were dark brown in color and slightly yellowish-brown fluorescent. Glucose and acid, with no amino acid added, was also dark brown in color and slightly yellowish-brown fluorescent. However, only those mixtures containing tyrosine or tryptophane remained fluorescent when diluted to several liters. It was estimated that the amount of fluorescence produced with glucose and tyrosine or tryptophane was about the same as obtained from one gram of egg albumin or wheat gluten.

Blue-fluorescent compounds have been found to be
closely associated with melanins in animal organisms (19).
It is well known that tyrosine and tryptophane are the only
amino acids involved in the production of melanins in na-
ture. However, when tyrosinase of potato juice was allowed
to act on tyrosine in buffered solutions, the resulting
brown solution was very weakly fluorescent (blue).

6. Extraction and concentration of the fluorescent material
of proteins.

Attempts to remove the fluorescent material from pro-
teins by dialyzing their solutions against distilled water,
using collodion or parchment membranes, were unsuccessful.
The fluorescent material is not dialyzed from neutral,
basic, or acid protein solutions. Dialysis of protein
hydrolysates, on the other hand, removes the fluorescent
material from the hydrolysate but does not separate it from
amino acids which also dialyze.

When proteins are salted out of solution with ammonium
or magnesium sulfate, the filtrates which are free from pro-
tein are not fluorescent. The fluorescent material is thus
shown to be securely bound to the proteins; perhaps it is
a constituent of the protein molecule.

Likewise, the extraction of protein solutions with
organic solvents such as ether, chloroform, butyl or amyl

alcohol, or benzene, did not remove significant amounts of fluorescent material from the proteins nor their hydrolysates. Amyl alcohol was the most efficient of these solvents in the extraction of fluorescent material from protein hydrolysates. When extracted by amyl alcohol in a continuous extractor for three days, about 10 per cent of the fluorescent material was removed.

The problem of the separation of the protein hydrolysates into their amino acid components is beset with many difficulties and numerous technics have been proposed. Using the Van Slyke technic for the nitrogen distribution of the hydrolyzed proteins, it was found that the fluorescent material is distributed in both the dibasic amino fraction and the monoamino fraction. Other proposed fractionations of amino acids, which involve precipitations in acid solution, cannot be used to concentrate the fluorescent material of the hydrolysates, because the precipitates formed in acid solution partially adsorb the fluorescent material. After several precipitations have been made from acid solution, most of the fluorescent material has been removed by such precipitates and recovery from these amino acid fractions is difficult.

The adsorption of the fluorescent material from acid solution was utilized as the basis of a method of concentration and purification of this material. Although it is

partially adsorbed by many inorganic precipitates formed in acid solution, lead sulfide was found to be quite efficient for its adsorption. However, large amounts of sulfide are required and the elution methods employed were not satisfactory for the recovery of the fluorescent material after the adsorption.

Norite "A", Cenco fuller's earth and English fuller's earth were found to be more satisfactory as adsorbants for the fluorescent material than lead sulfide or other inorganic compounds. Relatively small amounts of Norite "A" are required for the complete adsorption of the fluorescent material from protein hydrolysates. However, attempts to remove the material from the Norite "A" by elution methods were not very successful. It was found that mixtures of alcohol and ammonium hydroxide were quite efficient as elution reagents. After considerable experimentation, a mixture of 90 volumes of ethyl or methyl alcohol and 10 volumes of concentrated ammonium hydroxide was adopted as the most efficient elution mixture. With this mixture, only about 45 per cent of the fluorescent material could be recovered from the Norite "A".

Cenco fuller's earth is inferior to Norite "A" in its adsorption of the fluorescent material from protein hydrolysates, but only 70-80 per cent of the adsorbed material could be recovered by elution.

English fuller's earth was found to be the most satis-
factory adsorption medium for the concentration and re-
covery of the fluorescent material. The adsorption from
acid solution is quite complete and elution with the alcohol-
ammonium hydroxide mixture yields about 80-90 per cent
recovery of the adsorbed material. However, before being
used, the fuller's earth must be treated as described above
in the section dealing with the source and treatment of
materials; fluorescent material must be removed from the
fuller's earth.

The fluorescent material is not adsorbed by English
fuller's earth in basic or neutral solution, but excellent
adsorption takes place in strong acid solutions. Elution
is brought about only by alkaline reagents. A mixture of
90 volumes of either methyl or ethyl alcohol and 10 volumes
of concentrated ammonium hydroxide was found to be the
most efficient elution agent.

After numerous experiments, the method of concentra-
tion of the fluorescent material from protein hydrolysates
finally adopted is as follows: English fuller's earth is
mechanically stirred into the filtered acid hydrolysate
from about 100 grams of protein until the fluorescence is
removed from the solution. The solution is filtered and
the fuller's earth mechanically stirred into a liter of one
per cent hydrochloric acid and again filtered. This washing

process is repeated. Finally the fuller's earth is mechanically stirred into two liters of distilled water, filtered off, and again washed with distilled water. The fluorescent material is eluted from this washed fuller's earth by mechanically stirring it into liter portions of the alcohol-ammonium hydroxide mixture mentioned above and filtering. This elution is repeated until the filtrate is only weakly fluorescent.

The combined filtrates from the elutions are then evaporated to dryness under reduced pressure. The residue is thoroughly extracted and washed with 300-400 cc. of dilute hydrochloric acid (2 per cent). Another portion of English fuller's earth is stirred into this acid extract until the fluorescence is completely removed. (This requires less adsorbant than the first adsorption.) The fuller's earth is washed with dilute hydrochloric acid and distilled water as for the first adsorption. The fluorescent material is eluted by mechanically stirring the fuller's earth into 500 cc. portions of the elution mixture until the filtrates are only slightly fluorescent. The filtrates from the elution are evaporated to dryness under reduced pressure.

The material obtained in this manner is a small amount of dark brown, amorphous, non-fluorescent (in the solid state) material which is soluble in dilute bases or acids, and insoluble in ether, acetone, ethyl acetate, benzene,

toluene, chloroform or absolute alcohol. It is somewhat
soluble in methyl alcohol or 95 per cent ethyl alcohol.
It is soluble in amyl alcohol or acetic acid and is very
strongly bluish-green fluorescent in those solvents in
which it dissolves.

7. Properties of the fluorescent material from protein hydrolysates.

Since the fluorescent material was not obtained in
crystalline form, satisfactory purification has not as yet
been obtained. In fact, by the use of chromatographic ad-
sorption of the fluorescent material on Hydralo in a column
14 cm. high and 3 cm. in diameter, some evidence was ob-
tained to show that the material is composed of two com-
ponents. However, the evidence was not conclusive and more
work will have to be done before the fluorescent material
is obtained in a pure state.

Some of the properties of the material can be given.
It is not precipitated by protein precipitants, nor does
it give the color tests of proteins or amino acids. It is
not salted out of solutions by saturation with ammonium or
magnesium sulfate. Its fluorescence is not altered by
hydrolysis with sulfuric acid (20 per cent) for several
hours. It contains 20 per cent nitrogen but no sulfur.
It is not reduced by sodium hydrosulfite in basic solution

nor by zinc in acid solution. It is readily oxidized by
permanganate in acid solution.

When neutral solutions of the fluorescent concentrates
are injected into the peritoneal cavity of rats, no notice-
able reaction takes place. This indicates that the material
is not the fluorescent harman produced from tryptophane by
mild oxidation of the tryptophane-acetaldehyde complex. It
also indicates that the material is not a diketopiperazine,
since these compounds are toxic to animals. The fluorescent
material is excreted by the kidneys when injected into rats
and about 85 per cent of it can be obtained in the urine of
rats after intraperitoneal injections. It is quite slowly
excreted; 85 per cent can be recovered in the urine excreted
during the 48 hours following injections. However, increased
urinary fluorescence is noted for several days after the
injections.

The fluorescent spectrum of the material isolated from
protein hydrolysates was taken in order to determine whether
or not it contained line structure which might be used in
the identification of this material. The spectrum was also
taken to more clearly determine the color of fluorescence
and to compare its fluorescence with that of quinine bisul-
fate, thiochrome and lactoflavin.

The spectra were obtained with a Gaertner spectrometer
having a photographic attachment. The solutions were placed

in a quartz cell 2 cm. thick, 5 cm. wide and 6 cm. deep.
The outside of the cell was painted with black non-fluorescent
paint to eliminate reflected light, except for a window on
one side for the incident light and a narrow slit on the
adjacent side to allow the fluorescent light to enter the
slit of the spectrometer. Thus, the incident light from
the quartz mercury-vapor arc penetrated the solution in the
cell at right angles to the slit through which the fluores-
cent light entered the spectrometer. When unfiltered light
was used, the scattered and reflected light caused mercury
lines to appear on the spectrogram. However, with a filter
of 4 mm. thick, heat-resisting, red-purple glass, a very
small amount of light from the mercury arc entered the
spectrometer. The lines on the spectrogram due to reflec-
tion and scattering were very faint, even when exposures of
30 hours were made.

The wavelength standard used was the copper arc. It
was photographed using the same slit opening as was used
for the fluorescence spectra. The wavelengths were identi-
fied from the dispersion curve of the instrument.

Photograph No. 1 shows the fluorescent spectrum of
quinine bisulfate dissolved in 0.5 $\underline{N}$ sulfuric acid. The
standard copper arc for comparison and identification of
the wavelengths is also shown. The slit opening was 0.20
mm. and the exposure for quinine bisulfate was 27 hours.
The copper arc exposure was 6 seconds.

No. 1
Quinine bisulfate.
Standard copper arc.
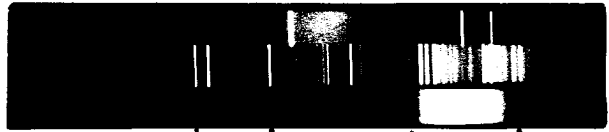
No. 2
Fluorescent material of
casein in dilute acid.
Standard copper arc.
Lactoflavin.

No. 3
Standard copper arc.
Thiochrome.(Hg. lines).

missing

No. 4
Fluorescent material of
casein in amyl alcohol.
Standard copper arc.

No. 5
Quinine bisulfate.
Standard copper arc.

Photographs of Fluorescent Spectra and Copper Arc.

Photograph No. 2 shows, (a) the fluorescent spectrum of the fluorescent material obtained from casein and dissolved in 0.5 N sulfuric acid, (b) the spectrum of the copper arc, and (c) the fluorescent spectrum of lactoflavin dissolved in 0.5 N sulfuric acid. All three were taken with a slit opening of 0.22 mm. The exposure time for the unknown material was four hours. The incident light was unfiltered light from a mercury-vapor arc. The mercury lines appear in the spectogram of the unknown due to incident light entering the slit of the spectrometer. The exposure time of the copper arc was 20 seconds and for lactoflavin the exposure time was two hours.

Photograph No. 3 shows the spectrum of the copper arc and the fluorescent spectrum of thiochrome dissolved in 0.1 N sodium hydroxide. The exposure time for thiochrome was 24 hours with a slit width of 0.22 mm. The copper arc exposure was 15 seconds.

The thiochrome was made from crystalline vitamin B_1 by oxidation with potassium ferricyanide, extraction of this product with butyl alcohol to remove the thiochrome, and then extraction of the butyl alcohol with dilute acid. The dilute hydrochloric acid solution, containing the thiochrome, was evaporated to dryness and taken up with a small amount of 25 per cent potassium hydroxide. This solution was extracted with chloroform until no fluorescence remained;

the chloroform solution was dried with anhydrous sodium sulfate. This solution was then evaporated to a small volume until crystals of thiochrome appeared. The product was recrystallized from chloroform. Thiochrome is only slightly fluorescent in acid solution, but very strongly blue-fluorescent in alkaline solution.

Photograph No. 4 shows the fluorescent spectrum of the unknown fluorescent material, from casein hydrolysates, dissolved in amyl alcohol. The exposure time was 24 hours and the slit opening was 0.22 mm. The incident light was filtered. The copper standard is also shown.

Photograph No. 5 shows the fluorescent spectrum of quinine bisulfate dissolved in 0.5 $\underline{N}$ sulfuric acid when filtered ultra-violet light is the exciting radiation. The exposure was 27 hours with a slit width of 0.22 mm. The standard copper arc shown in this photograph was slightly over-exposed (20 seconds exposure).

Attempts to obtain the fluorescent spectrum of the unknown material in basic solution failed because the fluorescent light given off is very faint.

The fluorescent spectrum of the material from protein hydrolysates does not contain line structure and therefore it has little value in identification of the fluorescent material. The fluorescent spectrum consists of a broad band in the blue-green region of the spectrum. This

spectrum resembles that of quinine bisulfate in acid solution, but is quite different from that of thiochrome or lactoflavin.

That the fluorescent material from protein hydrolysates is not quinine, is shown by the fact that quinine added to the hydrolysate can be recovered by ether extraction of the basic solution. This extraction does not remove the fluorescent material from the hydrolyzed proteins.

IV. DISCUSSION OF RESULTS

It was found that the proteins studied, namely, egg albumin, gelatin, blood fibrin, casein, wheat gluten, gliadin, glutenin, and zein, were all fluorescent in the solid state. The color of the fluorescence is very nearly the same in all of these proteins when excited with ultra-violet light of wavelengths 3100-4100 $\overset{o}{A}$. This observation is in agreement with the observations of Stübel (76). He found that many of the isolated proteins fluoresced with a blue color. Vlès (79) also found that proteins were excited to fluorescence. He used light of wavelengths from 2400 $\overset{o}{A}$ to the visible region and noted that the fluorescent color was the same for various proteins.

That the fluorescent material is not occluded or ad-sorbed is shown by the observation that various extraction processes or dialysis experiments failed to remove the fluorescence from the proteins. Since the fluorescence is destroyed by oxidation with nitric acid or by ashing of the protein, it is probably not due to inorganic material. This conclusion is also supported by the fact that the formation of many inorganic compounds in the protein or protein hy-drolysate solutions was never accompanied by an increased fluorescence, nor were any of the inorganic salt solutions resulting from blank or control experiments fluorescent.

The information obtained from the fluorescence of protein solutions also indicates that the fluorescence is very closely associated with the protein molecule and is organic in nature.

The data given in Tables I and II show that the fluorescence of proteins is greatly increased during acid hydrolysis, and that blood fibrin becomes more fluorescent than other proteins during hydrolysis in acids. This increased fluorescence of protein during hydrolysis was also noted by Pringsheim and Gerngross (64) who found that when gelatin was hydrolyzed with water at high temperatures, the fluorescence increase was proportional to the amount of hydrolysis. These authors did not study the effect of other methods of hydrolysis on the protein fluorescence.

The data of Tables I and II also show that the fluorescence of proteins reaches a maximum value when complete hydrolysis has occurred, and that proteins vary in the amount of fluorescence increase due to acid hydrolysis. Gelatin and zein give only small increased fluorescence compared to the increase of the other proteins during acid hydrolysis.

The data of Table III show that the fluorescence is characteristic of the protein molecule and not changed by purification of the proteins. Crude casein or gelatin gave the same amount of fluorescence as the treated or purified casein or gelatin. This also indicates that the fluorescence

is not due to inorganic impurities in the proteins.

The data obtained by the hydrolysis of proteins with alkali and enzymes (Tables V, VI, VII and VIII) indicate that acid hydrolysis increases the fluorescence of proteins much more than does alkaline or enzyme hydrolysis. This is probably due to the fact that acid hydrolysis destroys tryptophane to produce a fluorescent compound. Since the fluorescence of proteins is not greatly increased by alkaline or enzyme hydrolysis, the normal end-products of protein hydrolysis would not be expected to be very fluorescent. That this is the case, is shown by the examination of 19 known amino acids. When examined alone or in mixture, in the solid state or in solutions of various acidities and alkalinities, none of these amino acids was found to be noticeably fluorescent. A mixture of all of these amino acids did not show as much fluorescence as alkaline or enzyme hydrolyzed protein.

The fluorescence of certain amino acids has been reported in the literature. Stübel (76) claimed that tyrosine was weakly fluorescent and Vlès (79) found tyrosine, alanine, proline, and glutamic acid to be weakly fluorescent. He found arginine and histidine to be the most fluorescent of the amino acids examined. Glycine, cystine, and leucine are not fluorescent, according to Vlès. On the other hand, Kinnersley, Peters and Squires (46) found that histidine,

tryptophane, cystine, tyrosine, glycine and leucine were not fluorescent.

The observations on the fluorescence of amino acids reported in this thesis agree with the observations of Kinnersley, Peters and Squires, that amino acids are not fluorescent.

When proteins are hydrolyzed in strong acids, the increased fluorescence is probably due to the decomposition products of tryptophane which is destroyed under these conditions. Those proteins which are deficient in tryptophane are the only ones which do not give large amounts of fluorescent material during acid hydrolysis. Fibrin, which is rich in tryptophane, gives the highest fluorescence value. That tryptophane is responsible for the increased fluorescence is shown by the fact that when added to gelatin or zein, which are deficient in this amino acid, the amount of fluorescence produced by hydrolysis with strong acid is greatly increased. When this amino acid is added to these same proteins during hydrolysis with alkali, which does not destroy tryptophane, no increase in fluorescence occurs. Again, when mixtures of amino acids are boiled in acid the fluorescence does not increase, however, when tryptophane is present in this mixture fluorescence appears. The addition of other amino acids to gelatin or zein does not increase their fluorescence during hydrolysis.

The data of Table XI show that when tryptophane is added to casein there is not much increased fluorescence. This indicates that there are two factors involved in the fluorescence production due to tryptophane decomposition. It is possible that the unknown compound of the casein molecule, which reacts with tryptophane during hydrolysis, is completely used up by the tryptophane present in the casein and that added tryptophane cannot cause very much increase in fluorescence. It is believed that by use of the fluorescence increase during hydrolysis by acids, the tryptophane content of proteins could be estimated quite accurately, provided that an excess of the material with which it reacts is present.

A blue-fluorescent oxidation compound of tryptophane has been reported (35). This compound is an alkaloid of the cardiac-poison type. That the fluorescent compound of acid-hydrolyzed protein is not this same alkaloid is shown by the fact the two can be separated by ether extraction in alkaline solution; this removes the alkaloid. In addition, the fluorescent compound from hydrolyzed proteins does not have the physiological action of the alkaloid.

The fluorescence of proteins cannot be due to their vitamin content since various extractions failed to remove the fluorescent material and added vitamins did not increase the fluorescence of the proteins during hydrolysis.

Gortner and Holm (25) have shown that melanin (humin) formation is dependent on tryptophane and an unidentified constituent of the protein molecule which react during hydrolysis by acids to produce the insoluble black material. Fluorescence has also been shown to be closely associated with the formation of melanin in the skins of certain animals (19). The relationship between fluorescence and melanin formation was studied by preventing the formation of melanin during hydrolysis and also by the preparation of melanins with aldehydes and tryptophane. The results (Table XII) of prevention of melanin formation by the addition of stannous chloride to the proteins during hydrolysis indicate that fluorescence and melanin formation are somewhat related. The prevention of melanin formation decreases the amount of fluorescence produced, but does not prevent fluorescence production during hydrolysis. The melanin formed by tryptophane and aldehyde condensation in strong acids is fluorescent. Tyrosine also gives a black-colored melanin which has a blue fluorescence when this amino acid and glucose are boiled in acid. Other amino acids tested do not produce fluorescence with glucose.

When proteins are boiled with acetic acid, the fluorescence is greatly increased as is shown by the data of Table IX. This increased fluorescence with acetic acid is probably due to some addition compound of amino acids and

acetic acid, since several of the amino acids become fluorescent when boiled in acetic acid. In addition, when mixtures of acetic acid and hydrochloric acid are used, the fluorescence increase is not so pronounced. Also, the color of fluorescence is bright blue instead of the usual bluish-green.

The properties of the fluorescent material obtained from protein hydrolysates, by means of adsorption and elution, may or may not be the properties of the actual fluorescent material present, since satisfactory purification has not yet been obtained. Some evidence, obtained by chromatographic adsorption of the fluorescent material on Hydralo, indicates that this material is composed of two fluorescent components. One of these does not change fluorescence color with changes in pH, while the other changes from greenish-blue in acid solution to bluish-green in basic solution. However, this change in fluorescence color is not sharply defined.

The fluorescent spectrum of the fluorescent material from protein hydrolysates shows no line structure. It is a broad band in the blue-green region of the spectrum from about 4100-5300 $\overset{\circ}{A}$. The width and appearance of the fluorescent spectra of the other fluorescent materials, shown in the photographs, are in agreement with data given in the literature. It was found, by the use of filters of pure

organic liquids, that the incident light capable of produc-ing fluorescence of protein hydrolysates was composed of a band of light from 3400-3600 $\overset{o}{\text{A}}$.

V. CONCLUSIONS

As a result of the present investigation on the fluorescence associated with proteins the conclusions listed below have been derived.

1. The isolated proteins used in this investigation are all fluorescent in the solid state. They all fluoresce with a bluish-white color when examined in ultra-violet light of wavelengths 3100-4100 $\overset{\circ}{A}$.

2. These proteins are all fluorescent in solution. However, the color of the fluorescence in solution is more green than that of the solid protein.

3. Fluorescent material is not removed from protein suspensions or solutions by dialysis from neutral, slightly acidic or slightly basic solution. Likewise, extraction with various organic solvents does not remove the fluorescent material.

4. The fluorescence of proteins is destroyed by oxidation with strong nitric acid or by ashing the protein. The protein ash is not fluorescent in neutral, acid or basic solution.

5. The products of hydrolysis of proteins by proteolytic enzymes or alkali are only slightly more fluorescent than the original protein solution.

6. The color of fluorescence of protein solutions or products of hydrolysis is more green in basic solution than in acid.

7. Fluorescence was not evident in nineteen amino acids examined in filtered ultra-violet light of wavelengths 3100-4100 Å.

8. Hydrolysis of proteins containing tryptophane with strong acids greatly increases the amount of fluorescent material. The hydrolysis of the tryptophane deficient proteins, gelatin and zein, produces only very slight increase in the amount of fluorescent material.

9. Crude commercial proteins produce the same amount of fluorescent material, on hydrolysis with acids, as the same proteins in the purified state. The amount of fluorescence produced during hydrolysis with strong acids is not affected by the presence or absence of air.

10. Boiling proteins with acetic acid greatly increases the amount of fluorescent material. The color of the fluorescence is more blue then that of protein hydrolyzed with strong acids. The fluorescent material produced by the acetic acid and protein is not the same as that produced during the hydrolysis of proteins with strong acids.

11. The addition of tryptophane to proteins during hydrolysis with acids greatly increases the amount of fluorescent material. When added to proteins during alkaline

hydrolysis no increased fluorescence is observed. The addition of other amino acids to proteins during hydrolysis does not increase the amount of fluorescence.

12. When a mixture of amino acids (tryptophane omitted) is boiled with strong acids only slight fluorescence is noted. When tryptophane is added to this same amino acid mixture the amount of fluorescence is moderately increased.

13. The addition of vitamin B_1 and lactoflavin to proteins during hydrolysis does not increase the amount of fluorescent material produced.

14. Humin formed from glucose with tryptophane or tyrosine in acid solution is fluorescent with a bluish-green color. Other amino acids with glucose do not produce increased fluorescence.

15. Melanin formed from the tyrosinase of potatoes and pure tyrosine solutions is not fluorescent.

16. The fluorescent material from protein hydrolysates can be extracted by adsorption from acid solution by English fuller's earth and eluted from it with alcohol-ammonium hydroxide mixtures. The material thus obtained is extremely fluorescent in the blue-green region of the spectrum. This material has not been completely characterized. It is not harman (alkaloid derived from tryptophane).

17. The spectrum of the fluorescent material from protein hydrolysates is a broad band in the blue-green region

of the spectrum from about 4100 $\overset{\circ}{A}$ to 5300 $\overset{\circ}{A}$. This fluorescence is excited by light of wavelengths 3400-3600 $\overset{\circ}{A}$.

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VI. SUMMARY

A method of determining the relative amounts of fluorescence in protein solutions or hydrolysates has been developed and used in this investigation to study the fluorescence of proteins and their hydrolytic products.

The proteins prepared and studied were casein, wheat gluten, gliadin, glutenin, zein, gelatin and egg albumin. Hair and wool were also compared to the proteins.

These proteins were examined in ultra-violet light of wavelengths 3100-4100 Å. They all give a uniform bluish-white fluorescence in the solid state and a somewhat more green fluorescence in solutions. The fluorescence of these proteins is more green in basic solution than in acid.

Various methods of extraction with organic solvents and dialysis experiments failed to remove the fluorescent material from the solid proteins or their solutions.

Destruction of the proteins by oxidation with strong nitric acid or by ashing completely destroyed the fluorescent material.

The effect of purification of proteins on their fluorescence was studied. It was found that the degree of purity did not affect the amount or color of protein fluorescence.

The effect of hydrolysis of proteins by acids, alkalies and proteolytic enzymes on the fluorescence was determined. It was found that hydrolysis by proteolytic enzymes or alkali produced only a slight increase in the amount of fluorescence. However, hydrolysis with hydrochloric, sulfuric, perchloric or phosphoric acid produced large increases in the fluorescence of proteins containing tryptophane and only slight increases in those proteins which are deficient in this amino acid.

Acetic acid was found to increase the amount of fluorescence of proteins. However, the color of fluorescence produced by the acetic acid reaction with proteins is more blue than the fluorescence of protein hydrolysates.

Nineteen amino acids were examined in the solid state as well as in acid, neutral and basic solutions for fluorescence in ultra-violet light. None were noticeably fluorescent.

Amino acid additions to proteins during hydrolysis revealed that tryptophane was the only amino acid which increased the amount of fluorescence during acid hydrolysis. It did not affect the amount of fluorescence during hydrolysis with alkali.

The addition of vitamins which are capable of producing blue-fluorescent products when decomposed did not affect the amount of fluorescence produced during acid hydrolysis of proteins.

The production of humin by glucose and tyrosine or tryptophane produced blue-green fluorescence. The addition of other amino acids to glucose in acid solution did not increase the fluorescence. Melanin (humin) production by tyrosinase of potatoes and tyrosine did not produce fluorescence.

A method of concentration of the fluorescent material from protein hydrolysates was developed. It involves adsorption of the material by English fuller's earth and subsequent elution with alcohol-ammonium hydroxide solutions. Some of the properties of this fluorescent material are given. It is not harman (alkaloid derived from tryptophane).

The fluorescent spectra of lactoflavin, thiochrome, quinine bisulfate and the fluorescent material from protein hydrolysates were compared. These spectra are all broad bands. The spectrum of the fluorescent material from protein hydrolysates is in the blue-green region of the spectrum (4100-5300 $\overset{o}{A}$). The fluorescence of this material is excited by light of wavelengths 3400-3600 $\overset{o}{A}$.

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