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EFFECT OF CASTRATION AND TESTOSTERONE PROPIONATE ADMINISTRATION ON THE GLYCINE-2-C^{14} UPTAKE BY GUINEA PIG ORGANS

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CHAPTER I

INTRODUCTION

The problem of growth and its regulation, being concerned with one of the most peculiar if not exclusive patterns of living matter, has long since attracted the curiosity and challenged the ingenuity of scores of investigators.

This has resulted in a considerable body of information, which is still awaiting organization into a science, and in a wide series of experimental devices, each of which has the advantage of better focusing on one or another aspect of the complex problem.

In studying the mechanism by which androgens promote tissue growth, we have used the incorporation of glycine-2-C^{14} into body protein. In order to evaluate the significance and limitation of our experimental approach, a brief discussion of the whole problem will be given.

The Problem of Protein Biosynthesis

The structural and metabolic complexity of the systems capable of protein synthesis makes a schematization of the subject highly desirable. The transport of amino acids (AAc) across the cell membrane,

the energy donor(s) and the substrate "activation" and "organization" will be considered as phases at least logically separable. The thermodynamics and kinetics of the reaction will be considered next, and a discussion of the method of analysis and parameters of protein synthesis will follow.

Transport of AAc Across the Cell Membrane

A great deal of experimentation has been carried on to determine whether AAc are transferred into the cell by simple diffusion or by an active transport, both mechanisms being postulable a priori. The various techniques used include preparations of intestine, tissue slices, red blood cells, free tumor cells and bacteria. The results obtained have been various and sometimes contradictory.

Although the evidence for a simple diffusion mechanism is in several cases unquestionable (1-3) it has become apparent, since the early studies of Van Slyke and Meyer (4) that an active mechanism of transport or, to use Christensen's pictorial terminology, a "concentrative transfer" exists. Thus, it was observed that alanine, glycine and valine were absorbed at a rate not proportional to their concentrations outside the cell (5). Experiments with rats and with preparations of intestine demonstrated that L-isomers of AAc are absorbed at a rate greater than that of their D-analogs (6-16). These observations also showed that L-glutamic acid and L-aspartic acid were not transported against a concentration gradient. L-glutamic acid was not "actively" transported from the small intestine (17) but was by slices of guinea pig cerebral cortex (18).

Important results in this field are the ones reached by Cohen and Rickenberg (19-21), who have shown that E. coli can effect a high concentration of AAc across the surface. Thus, valine was concentrated 460 times across the cell surface. The concentrating mechanism is inhibited by DNP, and sodium azide. Equilibrium is reached in about 1 minute at 37°C. The mechanism is independent of protein synthesis and is not sensitive to Chloramphenicol or 5-methyltryptophan. The process is competitive in case of AAc of similar structure (valine, leucine, isoleucine), each one of the preceding AAc being capable of displacing the others from within the cell.

The relationship between AAc absorption and glucose absorption were studied and found largely independent (12).

All these results, though apparently confusing, point out that the mechanism need not be the same for all AAc or all tissues. Also, combinations of the two basic mechanisms should be postulated.

The concentrative transfer, demonstrated beyond any doubt, raises the questions of the energy supply. The observations that DNP (13), 4-deoxypyridoxine (14-22), anoxia and shock (16) inhibit the active transport, along with the fact that AAc are most readily concentrated if their amino group is not in the charged form (e.g. diamino acids), thus readily reacting to form Schiff bases (23) would indicate that both phosphorylation and Vitamin B_6 might be involved (24-29).

An exchange for intracellular potassium has been invoked as a mechanism for penetration of AAc into the cell (24-25). Thus, erythrocytes which have concentrated diaminobutyrate do so in exchange for K

which is ejected (24). Similarly, lysine, and arginine increase in the tissues of potassium-deficient rats (29), and it has been suggested that lysine may enter B. cadaveris freely, but is ejected in exchange for potassium by the transport device (30).

In the evaluation of experimental results, it must be borne in mind that the uptake of the AAc by the cell might vary independently with each of the following factors:

1. Concentration outside the cell.
2. Activity of the system capable of transferring across cell membrane.
3. Cellular reactions involving AAc.

This last factor, mainly because of practically impossible evaluation has, until recently, prevented sound conclusions. A significant step toward a better understanding of the mechanism and a more realistic appraisal of the experimental data is represented by the use, recently introduced by Christensen and co-workers, of α-aminoisobutyric acid (AIB) which undergoes concentrative transfer, but is not metabolized to any appreciable extent (31).

Another point worth considering, which the use of AIB together with that of α-methylolserine and α-methylserine (26) might help clarify, is the state of the AAc inside the cell. The possibility of a loose combination with some cellular component, suggested for the first time by Van Slyke and Meyer (4), has recently received new support. Kinetic studies have been performed (32-34) and either a free state or a loose combination has been claimed as proven. It is our belief that especially Heinz's results are not conclusive. This viewpoint seems to

be shared by Meister (35).

The exploration of endocrine influences on the AAc transfer which has just begun (31) appears to be a very promising field.

Although more than 45 years have elapsed since the first experiments by Van Slyke and Meyer, their conclusion that "a discussion of this problem would be at present premature," still carries part of its truth. It is unquestionable, however, that definite steps forward have been taken and a rigorous, rational approach reached.

Substrate "Activation" and Energy Donor(s)

The requirement for "activation" of AAc prior to peptide formation has long been recognized by various authors (36-38) on thermodynamical grounds. A comprehensive review of the problem has appeared recently (39). In 1955, Hoagland, Zamecnik and their group (40-41) described an amino acid dependent exchange of pyrophosphate with ATP according to the following scheme:

$$A\text{-}R\text{-}P\text{-}P\text{-}P + P^{32} - P^{32} \xrightarrow{AAc} A\text{-}R\text{-}P\text{-}P^{32} - P^{32} + P\text{-}P$$

In this system there is no net breakdown or synthesis of ATP. No mention is given by the authors concerning the fate of the amino acid involved. In other words, there is no mention of possible variations in the concentration of free amino acids.

The system is L-amino acid dependent, being a function of both concentration and number of AAc in the medium. AMP does not inhibit the exchange, nor exchanges, with ATP, indicating that no free AMP is formed.

Addition of hydroxylamine results in the formation of amino acid hydroxamates and in the appearance of an equivalent amount of

pyrophosphate. The formation of aminoacyl-adenylates as intermediates has been postulated, in spite of negative results, following search for their presence. It is interesting to note that the enzymes required for the reaction are the same "pH 5 enzymes" required for the incorporation of AAc into protein (vide infra). A preliminary fractionation would indicate that different enzymes are responsible for the activation of different amino acids. One disturbing point is that there is no direct relationship between the rate of exchange and the rate of hydroxamate formation for any particular amino acid.

Results consistent with the ones reached by the Harvard group have been obtained by Berg (92-99) in yeast. This author has shown that, similar to the activation of acetate, amino acid can be activated through formation of AAc-adenylates, both types of reaction probably representing examples of a more general mechanism concerned with biological endoergonic processes. It should be noted, however, that in spite of the fact that added synthetic aminoacyl adenylates were utilized with formation of ATP in presence of pyrophosphates, no "natural" free amino acyl-adenylates could be detected starting from free amino acids.

Further data along this line have been supplied by De Moss and Novelli (45-46) who have demonstrated the existence of this same process in bacteria. These authors have shown that no measurable changes in the concentration of ATP, pyrophosphates or AAc occur during the exchange. Analogous results have been obtained in various animal tissues (47-49) and plants (50-52). These last data are hardly compatible with the hypothesis that some AAc are tied in the formation of aminoacyl-adenylates, unless the concentration of these intermediates is very low

and they are converted to the final products with a speed many times the speed at which they are formed. On the other hand, this is difficult to postulate, in view of the fact that the exchange has been observed starting either with labeled ATP or labeled pyrophosphate.

Purification and separation of enzymes activating individual amino acids have been described (47-53).

There is, nevertheless, one disturbing point in this picture. Not all amino acids are able to catalyze the pyrophosphate exchange. The ones which have been found to be active, together with their relative activity, are listed below (45-46).

Tryptophan	20
Histidine	30
Phenylalanine	48
Methionine	122
Tyrosine	146
Valine	146
Leucine	168
Isoleucine	248

This fact, of course, offers the question whether the remaining amino acids are activated by some other mechanism.

Recently, (54-57) activation of all AAc has been described. For some of them, nevertheless, the experimental data reported are hardly significant when compared with their controls. Further confirmation of these experiments would seem desirable.

Mixed anhydrides of amino acids with other nucleotide polyphosphates besides adenylic acid have been described (58-60), but no other nucleotide has been found to be active in the pyrophosphate exchange.

The aminoacyladenylates, which are mixed anhydrides of carboxylic acids with substituted phosphoric groups, present considerable

interest, since substances of this class are known to react very rapidly with amino acids to form a peptide bond (61-69). In a neutral medium, aminoacyl adenylates will react at random to produce a wide variety of peptides.

Interesting considerations have been developed by Akoun, Simkin and Work (70) on the function of the enzyme involved in the amino acid activation. The fact that the complex seems to be very tightly bound to the enzyme, and the N-phosphoramidophenylalanine was found to be metabolically indistinguishable from phenylalanine, would suggest that among the functions of the enzyme there would be the protection of the amino group via a phosphoramid covalent bond. This protection of the amino group could be of real importance in regulating the otherwise indiscriminate reactivity of the compound.

The importance of aminoacyl adenylates as intermediates in protein synthesis recently has been challenged (71-73) by Ochoa, whose group has been able to isolate from the "pH 5 enzymes" of various microorganisms, an "amino acid incorporation enzyme" capable of incorporating amino acids into bacterial proteins and totally inactive in the "pyrophosphate exchange." This latter enzyme apparently can bypass the "activated AAc (Adenylates)" even though "the occurrence of some (other) kind of amino acid activation is indicated by the inactivity of the system under conditions in which oxidative phosphorylation cannot take place."

Recently, other ways of peptide formation have been found (74). This method involves the use of high temperature, and is at present

well beyond the realm of physiology. Their interpretation could nevertheless provide further insight into this complicated problem.

Organization of AAc into Protein Molecule

The question whether protein synthesis occurs by a stepwise condensation of smaller peptides, or whether by simultaneous condensations of AAc on a template has not received a satisfactory answer. The problem has been attacked variously, one of the most interesting approaches being the determination of specific activity in various fragments of the protein molecule following previous incubation with one or several labeled amino acids. This is on the reasonable assumption that a synthesis by simple terminal addition of the marked amino acid would result in accumulation of radioactivity in one end of the molecule, while a uniform labelling would indicate an "all or none" mechanism.

It should be mentioned, however, that unequal distribution of the label is incompatible with an "all or none" hypothesis only if a subsequent "exchange" of AAc between the already formed molecule and a pool can be excluded, and there is ample evidence (147) that this is not necessarily so.

Also, unnatural amino acids have been used in the attempt to decide whether incorporation of all AAc was simultaneously affected, or instead, if only the natural analog of the compound tested was involved in the inhibition.

Along these lines, Anfinsen, Steinberg and Vaugham (75-78) have investigated the biosynthesis of ovoalbumin by the hen's oviduct. The ovoalbumin, synthesized from $C^{14}O_2$, was cleaved enzymatically to

plakalbumin and peptides, and it was found that aspartic acid obtained from the peptides was labelled more heavily than was that obtained by hydrolysis of plakalbumin. Similarly, using C^{14} - amino acids, non-uniform labelling of glutamic acid, glycine, serine and alanine was shown. The authors conclude, on the basis of their results, that a stepwise condensation of AAc to form template-bound intermediates occurs.

In contradiction with the above results, it was found (79) that administration of labelled valine leads to uniform labelling of terminal and non-terminal valine obtained from rat hemoglobin. Also, Work and his group, studying the labelling of casein in a lactating goat, after administration of C^{14} - AAc, found equivalent distribution of the label among various protein fractions (80-82).

Velick and co-workers (83-84) have found that, after injection of five radioactive AAc, each amino acid in rabbit muscle aldolase was 1.8 times as active as the corresponding AAc from glyceraldehyde-3-phosphate dehydrogenase. Also, injecting phenylalanine-3-C^{14} and isolating phenylalanine and tyrosine, it was found that H-meromyosin, actin and the two glycolitic enzymes mentioned above were synthesized from the same AAc pool, while the specific activities of L- and H-meromyosin indicated that the two molecules were synthesized independently.

Later studies by Steinberg (85-86) have pointed out, on the basis of kinetic considerations, that non-uniform labelling can be shown only in short term experiments, longer incubations (more than 10 hours) always resulting in uniform labelling in spite of a possibly sequential synthesis.

Gehrmann and co-workers have reported a more rapid degradation

of C-terminal than non-terminal glycine, in collagen of rats prefed glycine C-14 over an eighty day period (87). On the other hand, the claim that N-terminal glycine from silk fibroin, obtained following injections of C^{14} glycine into body fluids of Bombix mori (88), was nine to ten times as radioactive as non-terminal glycine is of difficult evaluation because of obvious technical error.

Evidence that free peptides are not formed during protein synthesis has been supplied by Halvorson, Spiegelman and Hinman (89). These workers have shown that tryptozan, a potent tryptophan antagonist, inhibits the induction of maltase in yeast, without demonstrable accumulation of peptides. The same authors had reached comparable conclusions from the results of analogous studies with the use of p-fluorophenylalanine (90-92). It is difficult to see how the analog of one particular amino acid could prevent the formation of di - and tri-peptides from other, unrelated amino acids, if such peptides do indeed represent normal stages of protein synthesis. Hence, the negative outcome of the search for such peptides in inhibited cells suggests that they may not occur as intermediates in protein synthesis.

However, the demonstration that purine and pyrimidine analogues can be incorporated into polynucleotide structures during nucleic acid synthesis (93) raises the question whether amino acid analogues might similarly be incorporated into polypeptide structures during protein synthesis. It should be kept in mind that in the experiments conducted by Spiegelman's group, only the enzyme activity has been measured as a parameter of protein synthesis. It is entirely possible that a protein,

containing tryptozan or p-fluorophenylalanine instead of the natural analogues, could be formed and be void of enzymatic activity.

In support of this concept are the experiments conducted by Kidder and Dewey (94), who suggest that the inhibition of growth in Tetrahymena pyriformis might be due to the synthesis of protein containing the unnatural analog and void of indispensable enzymatic function. Also, Gross and Tarver (95) demonstrated the presence of ethionine in the protein of the same microorganism, after growth in presence of the analog. Again, Pardee and co-workers (96-97) have shown that 7-azatryptophan can replace tryptophan in the growth of a tryptophan-requiring mutant of E. coli, although such growth ceases after protein, RNA and cell count have approximately doubled, suggesting, according to the authors, the formation of inactive proteins by incorporation of the analog in place of tryptophan. The incorporation of azatryptophan was demonstrated by paper chromatography. The question whether proteins, made by bacteria grown in presence of azatryptophan, are biologically active was approached by determining enzymatic activities and bacteriophage formation. It was found that a number of inducible and constitutive enzymes were not formed (as measured by activity) in absence of tryptophan or in presence of 7-azatryptophan, tryptozan or 5-methyltryptophan. However, it was possible to show increased activity of serinedeaminase and ureidosuccinic acid synthetase in presence of 7-azatryptophan. Tryptozan gave similar results while 5-methyltryptophan was not capable of supporting growth.

Incorporation of p-fluorophenylalanine also has been reported. Munier and Cohen (98) have grown E. coli in presence of para-fluorophenylalanine and have shown that (a) the exponential type of growth

curve becomes linear after addition of the analog, (b) the increase of the bacterial mass is paralleled by increase in protein, (c) p-fluorophenylalanine can be demonstrated in the protein formed under these conditions, and (d) the induced biosynthesis of β-galactosidase is not modified. Analysis of the protein formed in such conditions, shows a decrease of the phenylalanine (23 per cent) and tyrosine (47 per cent), while normal amounts of valine and a normal leucine/isoleucine ratio are present. Presence of chloroamphenicol completely inhibits incorporation of p-fluorophenylalanine.

Additional information concerning the mechanism of protein biosynthesis (stepwise or template) are provided by nutritional studies, which have demonstrated that, with AAc mixtures, simultaneous presence of all essential AAc is required for optimal utilization (99-101). Further studies by Halvorson and co-workers, on the formation of adaptive enzymes in bacteria have led these authors to the conclusion that (a) "enzyme formation is mandatorily linked to the utilization of free amino acids" and (b) "the first stable intermediate on the way to an enzyme molecule is of such complexity as to demand simultaneous utilization of all amino acids" (102-103). Also, studies on β-galactosidase (104-106) in E. coli and the synthesis of amylase by pancreas slices (107) have again shown that all constituent amino acids are needed at the same time. The evidence that the same situation occurs during the synthesis of ferritin (108) also supports this general theory.

Summarizing the only data inconsistent with a stepwise synthesis of protein are those related to the lack of isolation of peptide

intermediates, all the others being capable of other explanations, besides the ones offered by the various authors. Nevertheless it is possible that, although peptide intermediates have not been identified, such intermediates have only transient existence. Until relatively recent times, the lack of isolation from tissue of free fatty acid of intermediate chain length was considered as an argument for a "template theory of fatty acid synthesis." Our list of naturally occurring peptides is still far from complete, and such other types of amino acid-containing compounds such as amino acid adenylates and other nucleotides containing amino acids, remain to be investigated. Tarver, one of the proponents of the template hypothesis, states that "one of the main advantages of the template idea is that it may be evoked at any stage in the process of synthesis, to fill in and banish the unseemly vacuum, until such time as new knowledge makes its revocation (or substitution) possible (109)." This opinion is shared by other authors (110-112), and represents indeed an equilibrated view of the problem.

If the hypothesis of a template is accepted, even with all the reservations suggested by what was said above, it will be easy to see that only two cellular components could satisfy the requirement imposed on a molecule that it be capable of transferring informations and organization to protein: cellular protein and nucleic acids. Nucleic acids are considered by Spiegelman (113) as the only possible sequence determining device.

From the studies on inborn errors of metabolism (114) on the heredity of the anthocyanin pigment of flowers (115), on the development of

eye pigment in Drosophila (116), and on auxotroph mutants in microorganisms (117-118), the idea emerged that synthesis of each enzyme and each specific protein is associated with and regulated by one gene. The success of the "one gene-one enzyme" theory on its applications to microbiological problems made it so familiar that investigators are often inclined to consider it an axiom rather than a working hypothesis. The more recent data obtained by Pauling and his group (119-120) on sickle cell anemia and those by Kalckar, Anderson and Isselbacher (121) on galactosemia have strengthened the already wide recognition of a relationship between protein and genetic material, and extended it to man. The appearance of sickle cell hemoglobin instead of normal hemoglobin results from a single gene mutation and consists in the replacement of one glutamic acid residue by one valine residue in hemoglobin, this being the only apparent difference in a polypeptide made of some 300 amino acid molecules.

The suggestion that nucleic acid plays a role in the synthesis of protein was first made about 1940 by Caspersson (122) and by Brachet (123). The evidence which supports this view has been reviewed recently by Hotchkiss (124) who has established nucleic acids as the genetic material responsible for transmission of information on synthesis of the individual protein.

From the data available, it is nevertheless difficult to establish the mode of action of DNA in protein synthesis, whether this would be a direct one or simply an indirect one (via RNA?).

The most direct approach to this problem has been the study of

the effect of enucleation (the nucleus contains practically 100 per cent of the cellular DNA) on the synthesis of cytoplasmic protein. Two unicellular organisms, Acetabularia mediterranea (a green marine alga) and Amoeba proteus (a protozoan), have been used widely for experiments of this type. Both these organisms, when cut into nucleated and non-nucleated halves, can survive for a considerable time.

Studies by Brachet and co-workers (125-127) have shown that the rate of incorporation of CO_2 or of glycine into proteins is the same in enucleated as in nucleated parts up to two weeks after cutting the alga and that net synthesis of cytoplasmic protein is independent of the nucleus. Also, formation of aldolase proceeds at a normal rate in the non-nucleated part of Acetabularia. Nevertheless, protein synthesis in Acetabularia cannot be considered completely independent of the nucleus, since between the twelfth and the fifteenth day after section, net synthesis of protein stops, although the fragments continue to survive for two months thereafter. It would appear that some substance produced by the nucleus and required for the synthesis of cytoplasmic material has been exhausted in the enucleated fragments.

With Amoeba proteus results are more difficult to interpret since after enucleation, the fragments rapidly lose their ability to move about and to catch prey. This results in starvation and surviving at the expense of their own stores. The experiments by Mazia and co-workers have shown (128-129) that the amino acid uptake is reduced, although less than protein synthesis, along with the loss of glycolysis and of the ability to use the stores of polysaccharides and lipids (130-132). It is little

wonder then that protein synthesis is decreased in such conditions, and it is difficult to say whether it can be ascribed directly to the absence of the nucleus.

Studies on mammalian reticulocytes, naturally occurring enucleated cells, have shown that the cytoplasm is capable of incorporating amino acids into its protein (133-134) and even to synthesize hemoglobin (135-137).

On the basis of these results, one can doubt whether the nucleus has any direct influence on the synthesis of cytoplasmic protein.

Concerning the synthesis of nuclear protein, it was found that, in isolated thymus nuclei, protein synthesis as measured by incorporation of C^{14} labelled AAc virtually ceases when DNA is removed from the nucleus, and that AAc uptake resumes when DNA is restored. The same authors (138-143) found that protein synthesis in nuclei is inhibited by DNAse and is insensitive to RNAse or Chloramphenicol, both of which are strong inhibitors of protein synthesis in many systems.

It should be mentioned, nevertheless, that in these studies, the "protein synthesis" was measured only by C^{14} AAc incorporation. The validity and limitation of this approach will be discussed in a subsequent section (vide infra).

The requirement of DNA for the incorporation of AAc into proteins by disrupted Staphylococcus aureus has been clearly established by Gale and Folkes (144-145). Further experiments by the same authors have indicated that the specificity for DNA is not absolute, since fragments not yet identified, obtained by RNAse digestion of RNA and which do not

contain nucleotides are capable of restoring the full activity of the system (146-150). Also, Spiegelman (113) was able to remove all of the DNA from osmotically shocked protoplasts of Bacillus megaterium without inhibiting enzyme synthesis.

A thymidine-requiring strain of E. coli (89) has proved useful in exploring the relationship between DNA and other cell constituents. Formation of proteins, RNA and even induced enzymes occur when DNA synthesis is prevented by the omission of thymidine from the medium (151-153). If this proves that "concomitant synthesis of DNA" was not a limiting factor in the above experiments, it does not give any information concerning the relationship between the preformed DNA and any one of the parameters tested.

From the data gathered it seems evident that, even though it is likely that the genetic material regulates enzyme and protein synthesis, nevertheless, this regulation is not a direct one. The possibility of an "intermediate" regulator exists. RNA seems to possess all the required qualifications for this function.

The evidence that RNA could function as a template for protein synthesis was reviewed in 1955 by Brachet (154), one of the originators of the theory. Such evidence seems relatively indirect, describing either concomitant arrest in both RNA and protein synthesis, or effects of RNA on amino acid incorporation into protein, whose interpretation is by no means clear (vide infra). It should also be noted that Brachet himself (154) states that "there is no reason to believe that RNA alone plays a part in protein synthesis: It is quite possible that the whole granule,

the microsome, is the active agent."

Some experiments have been reported, nevertheless, which can be explained more easily by the assumption that RNA is the "template." So Gale and Folkes (144-145) have shown that preparations of disrupted Staphylococcus aureus require both RNA and DNA for the synthesis of glucozymase and catalase, both being constitutive enzymes. However, in the same preparations, induced synthesis of β-galactosidase was independent of RNA, as long as purines and pyrimidines were present, but was dependent on the presence of DNA. The authors suggest that constitutive and adaptive enzymes might require different "templates" for their synthesis (147).

At the same time, such preparations demonstrated that intact RNA was less efficient in restoring enzyme synthesis than RNA fragments obtained by digesting RNA with RNAse (147). The chemical nature of such fragments has not been identified, but it is apparent that each fragment, which is relatively specific for the incorporation of a particular amino acid, is free from nucleotides. A method of partial purification for one of these "amino acid incorporating factors," the one active toward glycine, has been described recently by the same authors (149). It is interesting that such a factor, 10^4 times as active as RNA in incorporating glucine, also promotes incorporation of adenine into the preparation's nucleic acid (150), thus offering support to the hypothesis of a simultaneous synthesis of both protein and nucleic acids (vide infra).

The observations, of course, would make the RNA-template hypothesis extremely unlikely. The presence of small fragments, in this latter

case containing nucleotides, responsible for the incorporation of specific AAc's has been confirmed, with a completely different approach, by Zamecnik in liver microsomes (155).

Ribonuclease has been widely used to show whether intact RNA is required for protein synthesis. Thus, Spiegelman and Landman (156), under carefully controlled conditions were able to remove a large part of RNA from protoplasts of B. megaterium without destroying these structures. They observed that destruction of RNA resulted in complete inhibition of enzyme synthesis. Also, using protoplasts of B. megaterium submitted to a controlled osmotic shock, Spiegelman (113) succeeded in removing selectively either RNA or DNA. The effect of these treatments upon enzyme synthesis led him to conclude that physical integrity of the RNA molecule is of paramount importance for enzyme formation. It is not certain, nevertheless, whether RNAse always exerts its inhibitory effect on protein synthesis by splitting RNA. Thus, in onion roots (163) and in ascites cells (164), RNAse first stimulates RNA synthesis. Evidence is at hand to show that RNA formed in such conditions contains an excess of pyrimidines (165). Also, basic proteins have been shown to inhibit amino acid incorporation into tissue protein, even though not at the same rate as RNAse (166), and complexes with RNA, acting as RNA inhibitors, have been postulated (167).

Other observations, bearing on the importance of intact RNA for protein synthesis, are those concerning the effects of analogues of the natural purines and pyrimidines. When RNA from plant viruses has incorporated thiouracyl or azaguanine, the infectivity of the viruses was

found to be considerably reduced, and it is clear that the abnormal RNA is unable to fulfill its function (156-162). It has also been shown that B. cereus can substitute as much as 23 per cent of its RNA guanine with azaguanine (168): In such conditions growth is only partly inhibited, but the synthesis of several enzymes is completely arrested (169-170).

Possibly the most convincing data concerning the relationship between RNA and protein synthesis are offered by the studies performed with viruses. It has been shown that RNA extracted from several viruses is capable of inducing the production of specific proteins by the host. This establishes that specific RNA's are capable of inducing protein synthesis. The evidence in this field has been reviewed recently by Schramm (171), to whom the reader is referred. This situation may not be restricted to viruses. In fact, several laboratories have reported similar results with bacteria. Thus, copper resistance could be transferred to a non-resistant strain from adapted strain by adding to the former RNA extracted from the latter (172). Also β- galactosidase (173), glucokynase (174), and penicillinase (175) could be induced by transfer of RNA from an adapted to a non-adapted strain. These results are all of the utmost interest. Limitations to their interpretation should, nevertheless, be sought in the questionable reproducibility and in the lack of appropriate methods capable of yielding homogenous preparations of RNA.

Several observations are not immediately consistent with the hypothesis that RNA serves as a template for protein synthesis. Thus, Hokin and Hokin (176-187) have found that stimulation of the synthesis of pancreatic amylase was not accompanied by an increase in RNA content

or by an increased P^{32} incorporation into RNA. Analogous results were found by Farber and co-workers (178), but one must remember that in this system, protein synthesis is inhibited by chloramphenicol (179).

An interesting point is the question whether preformed RNA is necessary and sufficient for the regulation of protein synthesis, or whether simultaneous synthesis of both RNA and protein is an obligatory pathway.

In 1953, Gale and Folkes (180) had observed that a supplement of purines and pyrimidines stimulates protein synthesis in S. aureus. This observation was later confirmed by Creaser (181). Also, using pyrimidine-requiring mutants of E. coli, Pardee observed (182) that induced biosynthesis of enzymes was possible only if exogenous pyrimidines were supplied, except in particular cases in which at least partial breakdown of cellular RNA could be demonstrated. This dependence of enzyme neoformation on nucleic acid precursors rather than on preformed nucleic acid was shown also by Spiegelman and co-workers (103).

Attempts have been made to follow directly RNA synthesis during enzyme formation, and net increase in RNA content has been shown to parallel the induction of enzyme (183-184).

Extensive studies by Gale (144-145) with preparations of disrupted S. aureus, and by Webster with pea seedlings (185-186) are best explained by assuming simultaneous synthesis of both RNA and protein. The evidence has been summarized by Gale (146-147), and a chemical mechanism for such a process has been proposed by Michelson (187-188).

In discussing the evidence for or against the possibility that RNA

might function as a template, only the papers in which net protein synthesis was demonstrable have been presented. The ample evidence in favor of a relationship between nucleic acids and incorporation of labelled precursors into protein will be discussed now. The reasons for this separation will soon become apparent.

The first studies on the incorporation of AAc into tissue protein were published by Schoenheimer and his group more than 15 years ago (189-196). The authors demonstrated that amino acid labelled with N^{15} and/or deuterium were incorporated by peptide bonding in tissue protein. Their technique consisted in separating tissues into a tricholoroacetic acid-precipitable fraction and a soluble fraction. The interpretation that the incorporation could represent either de novo synthesis or replacement as well was advanced.

Since the appearance of these papers, the publications in this field have grown so numerous to defy any attempt to review the subject with completeness. Several reviews have appeared recently (197-198), where most of the data can be found. Here, only data pertinent to our discussion will be presented.

The question whether the labelled amino acid(s) is (are) incorporated by peptide bond or attached to the protein by some other kind of bond has received wide attention. In a series of excellent papers, Borsook (199) has shown in a wide variety of experimental conditions that the labelled residue cannot be removed from the protein by drastic washings, by solution and reprecipitation of the protein or by any other treatment which did not hydrolyze the peptide bond. Using a method capable of

hydrolyzing such a bond, it was found that the label was released at the same rate as the non-labelled amino acid, and that it was present in a wide spectrum of peptides of intermediate length. These results have received wide confirmation (197).

The distribution of the label among various tissues has been widely studied. It was found that certain tissues (parenchimatous tissues, stomach, intestine, etc.) incorporate the label at a much higher rate than e.g. muscle. Furthermore, the maximum labelling was reached by different tissues at different times. In the majority of these studies, no effort was made to isolate particular proteins; instead the label content of the whole "protein fraction" was measured. It is not unlikely that, within the same cell, different protein will take up the label at quite different rates.

Turnover rates have been calculated on the basis of these results (see the review by Tarver (109)). Generally speaking, these rates have been calculated on the assumption that the incorporation of AAc follows a first order mechanism. This assumption is entirely arbitrary. A more generalized mathematical treatment of the problem has been given (200-204) with little practical advantage, since most of the constants appearing in the new equations are beyond the possibility of experimental measurement.

It should be considered now whether such incorporation takes place as the result of protein synthesis or through some other process, and, in this latter case, which relationship exists between the "incorporating pathway" and protein biosynthesis. The discussion of the validity of the conclusion, drawn from results related to one of the two

phenomena and applied to the other, will thus be a natural corollary of our analysis.

The evidence is particularly hard to evaluate due to the variety of the experimental approaches adopted. Nevertheless, two general types of experimental devices can be identified, most of the papers in this field falling in one or another of two major groups. In the first group of experiments, (Gale's condition 1 (147)), preparations were incubated with one single amino acid (in addition to a source of energy, salts, buffers, etc.), while the whole set of indispensable AAcs were present along with the labelled one in what we consider as the second group (Gale's condition 2 (147)).

It has been shown that incorporation of AAc takes place in both conditions, and we know that condition 1 is not associated with protein synthesis, the simultaneous presence of all the indispensable AAcs being a "condition sine qua non" for that process to take place (145).

Furthermore, it has been shown (145) that the rate of incorporation of a single amino acid varies with the experimental conditions in a manner which is not correlated with protein synthesis. Also, the presence of an inhibitory analogue of any specified amino acid, while inhibiting protein synthesis, does not prevent the incorporation of a second amino acid (205-207). Also, the course of incorporation of AAc under conditions 1 and 2 appears to be quite different. Thus, while in condition 1 incorporation starts at a faster rate to reach equilibrium in about 60 minutes, the incorporation which can be observed under condition 2 proceeds linearly for several hours, no equilibrium being reached over the period

tested (about 7 hours). Furthermore, addition of unlabelled amino acid, in the presence of a source of energy, brings about the release of some of the label (144; 145; 206; 207) under condition 1.

A careful evaluation of the above-mentioned facts indicates that conditions exist (condition 1) consistent with label incorporation but not with protein synthesis. Until otherwise proven, incorporation of labelled AAc *per se* cannot be taken as a parameter of protein biosynthesis. The question nevertheless remains whether any relationship exists between the two processes, since so far, most of the inhibitors tested which affect amino acid incorporation also inhibit protein synthesis.

In order to clarify this important point, an impressive amount of experimentation has been carried on during the last few years, with very interesting results. Thus, Zamecnik and Keller (208) have shown that gentle homogenization of rat liver with a loosely fitting pestle yields a cell-free preparation capable of incorporating AAc. Centrifugal fractionation of the preparation showed that (a) mitochondria do not incorporate label to any appreciable extent, (b) both microsomes (105000 x g precipitable fraction) and supernatant alone are unable to incorporate C^{14} - AAc, and (c) full incorporation is restored when microsomes and supernatant are combined. Furthermore, it was found that the supernatant preparation was capable of activating AAc (*vide supra*, also 208-209). Further fractionation of microsomes by the use of sodium deoxycholate showed that the deoxycholate insoluble fraction, which contains about 90 per cent of the nucleic acid originally present in the microsomes, is

the first one to be labelled after administration *in vivo* of C^{14} - AAc. Labelling of this fraction is readily followed by the transfer of the label to the deoxycholate - insoluble material, which contains mostly microsomal protein (210-213). The same group has further purified the supernatant fraction, from which the so-called "pH 5 enzymes" have been isolated. Such enzymes were found (a) to contain relatively short polynucleotides (labile RNA), to be possibly the first fraction to incorporate labelled AAc, (c) to represent a set of enzymes each of which is responsible for the incorporation of a given amino acid, (d) to require specifically ATP, (e) to be the same enzymes capable of "activating amino acids," (f) to be able also of catalyzing the incorporation of C^{14} nucleoside-triphosphates of cytosine, adenine and, to a smaller extent, uracyl as end-molecules of the RNA chains present in the enzymes themselves (213).

Similar results were obtained using a slightly different preparation and fractionating the microsomes with increasing concentrations of pyrophosphates, by Sachs and his group (214-217), and by Shimkin and Work (218).

In partial contrast with the above experiments, McLean and his groups (219) have reported that muscle mitochondria are able to incorporate labelled AAc (it should be remembered that in the previously quoted experiments liver mitochondria were inactive), and that this property is not due to contamination with microsomes.

It is of interest to note that the incorporation into "pH 5 enzymes" is partly under the control of Vitamin B_{12} (220). Insulin has been reported to influence the incorporation of AAc into liver microsomes

(221), and at least part of this effect does not involve glucose transport (222).

All these results indicate a strict relationship between energy donors, free amino acid, protein and nucleic acids. The relationship between RNA content of the ribonuclein particle and rates and time-sequence of incorporation would indicate an unquestionable regulatory action of RNA on the incorporating mechanism. To extend this to the process of protein synthesis in itself appears to us, in view of the considerations illustrated above, arbitrary. As final data to illustrate that the two processes should be considered, for the time being, as parallel and largely independent of each other we should remember that, so far, no net protein synthesis has been achieved in the acellular systems used so brilliantly to illustrate the relationship between nucleic acids and incorporation of AAc (57).

A new interesting point, concerning the specificity of nucleic acid in their presumed function as regulators of protein synthesis (we should speak of incorporation and not of synthesis), is brought by a recent paper by Allfrey and Mirsky (223) who have shown that incorporation of labelled AAc into isolated calf thymus nuclei, decreased after removal of DNA, can be fully restored by addition of polyanions (pyrophosphate, polyethylenesulfonate, heparin, chrondroitinsulfate, polyadenylic acid), but not of polycations (protamine, polylysine). The significance of polyphosphates for the development of certain lower organisms has been shown by Belosersky (224) while Regelson has demonstrated the ability of certain polyelectrolytes to inhibit tumor growth

(225-226). These results suggest a relationship between AAc incorporation and electric charge.

From this survey it appears that, if there is much to suggest some relationship between nucleic acids and protein synthesis, very little data exist to indicate the form of such a relationship. Since DNA seems to carry the genetic information from one generation to another, it has been assumed that it must provide physically defined surfaces and patterns required for the synthesis of such a specific molecule as the protein molecule. To date, we have no definite proof that nucleic acid preparations are homogeneous, or that nucleotides follow each other in a definite sequence. This has been generously assumed but never approached experimentally.

A very interesting effort has been made by Gamow (227-229) to provide a mechanism for the transfer of information from a four digit system (the nucleic acids) to a twenty digit system (the protein). Nevertheless, it should be remembered that, although in Gamow's scheme combination of AAc with nitorgen bases is assumed, no physical or chemical basis for such a combination is offered (230). As a matter of fact, a careful examination of the DNA molecule, to see if any part of it might serve as a template for protein production, does not yield encouraging results. There is no reason to believe that the sugar and the phosphate part of the molecule is not completely regular (230). Since there are no breaks and branches in the phosphate ester chains, their configuration lacks the structural basis for exerting even such effects as the steric effect. One is therefore forced to localize all specificity in

the nitrogenous bases. These lie fairly well exposed to outside influence at the bottom of two helical grooves in the molecule. The surface of these grooves is relatively smooth, interchange of purine by a pyrimidine or of a hydrogen by a methyl or amino group causing a maximum variation in the position of the bottom of the groove of not more than 1.5 Angstrom (230). In other words, the difference of volume between the various purines and pyrimidines is much less than the difference in volume between the amino acids, leaving therefore, no obvious sign of template action in terms of space filling.

Furthermore, the difference in reactivity between the nitrogen bases, measured by the relative ease of substitution, etc., is relatively small. In addition to that, the most reactive groups are hydrogen bonded to link the bases in pairs. Also, the difference in number of hydrogen-bonding sites in the two pairs of bases is small. Furthermore, only one out of the four bases has one free amino group, the one which occurs in guanine on the shallower of the two grooves of the molecule.

To add to the uncertainty about the possible role of nucleic acid per se as templates, it must be pointed out (70) that, when bacteriophage nucleic acids penetrate bacteria, they undergo rapid depolymerization. However, the situation is not completely hopeless if we consider the whole ribonucleoprotein as the active unit. In this light, Brachet's statement (154) and Allfrey and Mirsky's results become of easier interpretation.

In concluding this survey it should be said that, since so far we have no methods for the purification of nucleic acids, any hypothesis

which would require the determinations of a sequence in a molecule whose weight is of the order of 6×10^6 or more is, for the time being, beyond the possibility of experimental support. The value of a theory, however, depends also on the amount of work it is able to stimulate. The amount of work stimulated by the above hypothesis has been plentiful.

Thermodynamic and Kinetic Considerations

The application of chemical and physical laws to the interpretation of biological mechanisms, blessed so far with a wealth of substantial contributions to the understanding of what is possibly one of the most dramatic challenges to the human mind, has developed containing the implicit assumption that no basic difference exists, both at the microscopical and macroscopical level, between the behavior of atoms and molecules while integrant parts of biological systems, and their behavior in the inorganic world.

The application of exact thermodynamical and kinetic criteria to the description of biological systems represents probably the most effective approach to test the validity of the above assumption.

The terminology and notations used in this section will be the classical one of Physical Chemistry, to textbooks of which the reader is referred (231-234).

Considering the reaction:

$$n\ AAc \longrightarrow {}^{m}\ Peptides + p\ H_2O$$

we know from thermodynamical considerations that:

$$1\ n\ K = \frac{-\Delta F}{R\ T}$$

where K is the equilibrium constant, R the gas constant and T the

temperature expressed in absolute degrees.

Since $F = F\ (T, P, \mu_i)\ V, S, \mu_j$, we can see that the relative amount of synthesis can easily be predicted, once ΔF^o is known, for any given T. P, and μ_j. In Table 1 (reproduced from Borsook (235)), these values are shown for a number of reactions. From these data, it is easy to see that, in the formation of dipeptide from its constituent amino acids, there is such a strong gain in free energy that under ordinary conditions spontaneous synthesis occurs only to a negligible degree.

It is also interesting to note how small is the effect of the concentration of the reactants on the degree of synthesis, at least at the concentration calculated. It should be remembered that concentrations of AAc in blood are of the order of 10^{-3} or less. At these concentrations, spontaneous synthesis would be negligible even in the most favorable cases listed in Table 1.

Of course the data on the active transfer of AAc into the cell would indicate that concentrations inside the cell wall could be considerably higher. It is entirely possible that intracellular loci exist (Microsomes?) in which the concentration of AAc may be so great to allow some spontaneous synthesis. Of course the solubility of AAc will be a limiting factor to how concentrated the solution may be.

It is a well known fact that the yield of a reaction will be increased if the product will be removed "sufficiently fast" or, which is equivalent, if it is "insoluble enough."

In Table 2, the effects of assumed solubility of the product have been calculated according to the usual thermodynamical methods, for two reactions.

TABLE 1 *

FREE ENERGY OF FORMATION OF SOME SMALL PEPTIDES, EQUILIBRIUM CONSTANT AND DEGREE OF SYNTHESIS BY MASS ACTION AT DIFFERENT INITIAL CONCENTRATION OF REACTANTS

Reaction (H_2O Omitted)	ΔF at 37.5°C cal.	Equil. Const. K x 10^3	Per cent Synthesis at Equilibrium at Initial Concentration of Reactants		
			0.1 M	0.01 M	0.001 M
Alanine + Glycine Alanylgycine	4130	1.25	4×10^{-3}	10^{-4}	10^{-5}
Aspartate + NH_4 + Asparagine	3460	3.67	4×10^{-2}	4×10^{-3}	10^{-3}
2 Glycine Glycylglycine	3590	2.99	3.3×10^{-2}	3.4×10^{-3}	10^{-3}
Leucine + Glycine Leucylglycine	3315	4.67	4.2×10^{-2}	5.3×10^{-3}	10^{-3}
Benzoate + Glycine Hippurate	2630	1.42	1.4×10^{-1}	1.4×10^{-2}	1×10^{-3}
Benzoate + Glygly Benzoylglygly	1100	15.64	1.5	1.6×10^{-1}	9.5×10^{-3}
Benzoyltyrosine + Glycineamide Benzoyltyrosyl-glycineamide (BTGA)	361	55.82	5.0	5.5×10^{-1}	58.0×10^{-3}

* Reproduced from Borsook (235) by kind permission of the author.

TABLE 2 *

DEPENDENCE OF EQUILIBRIUM DEGREE OF SYNTHESIS OF A DIPEPTIDE ON THE SOLUBILITY OF THE DIPEPTIDE, INITIAL CONCENTRATION OF THE REACTANTS AND EQUILIBRIUM CONSTANT

Equilibrium K	Assumed Solubility moles/liter	Per Cent Synthesis at Equilibrium Initial Concentration of Reactants		
		0.1 M	0.01 M	0.001 M
0.5582 (Synthesis of BTGA)	0.05	4.5	0.57	0.058
	0.005	9.7	0.57	0.058
	0.0005	65.0	0.57	0.058
0.001247 (Synthesis of Alan. Gly.)	0.05	1.6×10^{-2}	8×10^{-4}	10^{-5}
	0.005	1.6×10^{-2}	8×10^{-4}	10^{-5}
	0.0005	1.6×10^{-2}	8×10^{-4}	10^{-5}
	0.00005	1.6×10^{-2}	8×10^{-4}	10^{-5}
	0.000005	36.5	8×10^{-4}	10^{-5}

* Reproduced from Borsook (235) by kind permission.

It can be seen from this table that, in the particularly favorable case of BTGA, at a high enough (0.1 M) concentration, the per cent synthesis jumps from 4.5 per cent to 65 per cent if the assumed solubility of the product is reduced a hundredfold. In the less favorable case of AG, the solubility of the product has to be below 10^{-5} M, well below the solubility of $BaSO_4$ or Ca oxalate in water, before insolubility of the product will have any effect on the degree of synthesis. Whether these conditions are met *in vivo* is rather questionable.

A third point worth mentioning, and illustrated by Table 1, is the influence of the configuration of the molecule on ΔF^{o} for the reaction. Thus, there is a difference of 800 cal. between the ΔF^{o} of alanylglycine and of leucylglycine. Also, the synthesis of benzoylglycine (hippuric acid) requires 1000 calories less than the synthesis of glycylglycine. The reaction is even less endoergonic if benzoic acid reacts with glycylglycine instead of glycine.

It is furthermore interesting to note that, *ceteris paribus*, to form a peptide from two zwitterions represents a more endoergonic process than to form a peptide from simple ions. The example of ΔF^{o} for benzoylglycine as compared to ΔF^{o} for glycylglycine will suffice to illustrate this point. Consistent with this view is the observation that, when two zwitterions react, the reaction will be less endoergonic the farther apart the non-reacting groups. This point is illustrated by the examples of ΔF^{o} for benzoylglycine and benzoylglycylglycine (from benzoic acid and glycylglycine).

If both the reactants are ions of opposite charge, the energy requirement for the reaction will be even smaller.

It is opportune to note here that (vide supra), assuming that activation of AAc plays a role in the formation of peptides, one of the functions of the "activating enzymes" is that of "protecting" the amino group of the amino acid, thus transforming a zwitterion into a simple ion. The importance of this step has already been discussed.

These considerations, if developed to their ultimate conclusions, would point out that, per peptide bond formed, the largest expenditure of energy is met in joining together the first two amino acids, the "cost" of synthesis, per peptide bond formed, decreasing with the increase in length of the peptide chain. In this regard, it is highly interesting to note that the average ΔH^{o} per peptide bond hydrolyzed from β-lactoglobulin by pepsin is about 1300 cals. The products of the reaction are polypeptides of varying length. It is known, on the other hand, that the average ΔH^{o} of formation of a dipeptide is between 3000 and 4000 cals., very close to the corresponding value for ΔF^{o}. If we assume that, during the hydrolysis, ΔS^{o} per peptide bond is constant and independent of the chain length, a reasonable assumption since the variation in rotational, translational and vibrational degrees of freedom is grossly independent of the chain length, at least as long as both reagents and products are asymmetrical molecules, we are led to conclude that ΔF^{o} for the hydrolysis of a bond located inside a polypeptide is smaller in absolute value than the corresponding one obtained from the hydrolysis of the same bond in a dipeptide.

What the energy requirement for a "simultaneous" formation of all peptide bonds present in a protein would be is hard to predict. It appears, nevertheless that, if all AAc are present as zwitterions - and

there is no definite proof that this is the case -, the "all or none" mechanism would be considerably more expensive, energy-wise, than the "stepwise mechanism." The teleological implications of these considerations are left entirely to the reader. It should be added, however, that no definite evidence exists that transpeptidation plays a role in protein synthesis (236-237).

The discovery of the energy supply ("labile" pyrophosphate bond of ATP) and of the activated intermediate has represented a considerable progress in the understanding of the mechanism by which AAc can form peptide bonds. This, unfortunately, is only a partial answer, since two elements are characteristic of a protein molecule: Its amino acid composition and the amino acids' sequence and steric configuration.

Also, thermodynamics only tells us whether a reaction is "spontaneous," also called "natural," or not. No predictions can be made, on purely thermodynamical grounds, concerning the rate of reactions.

The absolute rate of protein synthesis, is, at present, largely unknown. Approximate calculations can be obtained from the rate of growth of young animals, bacteria or tissue cultures. The values obtained this way refer only to the rate of net protein synthesis, whose importance is extreme from a more "biological" standpoint, but whose importance in view of interpreting the chemical mechanism of the reaction need not be as great.

Also, data of undisputed interest can be obtained by measuring the uptake of labelled precursors. These data are, nevertheless, of rather difficult interpretation (vide supra).

The concept of a rapid protein turnover, originated with the work

done by Schoenheimer and his associates (189-196) and recently challenged by studies conducted by Spiegelman (238) and by Monod (104-105), has undergone various transformations and has existed under a discrete range of enunciations. If incorporation of AAc, even only under the restrictions imposed by Gale's 'condition 2' is taken to be an adequate parameter of protein synthesis and/or turnover, there would be no doubt that the process is one of the fastest in biology. This immediately raises two problems.

The first is a more thermodynamical one, in as much as it is concerned with the energy supply. It is known that the synthesis of a peptide bond requires on the average 3.5 Kcal. per mole. Since not all, and probably not more than 60 per cent of the energy liberated in the breakdown could be reused for synthesis (second law of thermodynamics), a considerable wastage of energy would take place.

It is almost impossible to calculate the energy involved, due to lack of adequate experimental data. A very crude calculation can be made as follows. In a 70 kilogram man, there are at least 10 Kg. of protein. Assuming the average molecular weight of AAc to be 200 g. (which certainly is in excess of the true value), we would have about 50 moles of peptide bonds, requiring close to 180 Kcal. In many instances, it has been found that label reaches its maximum incorporation within 6 hours. This would mean that, whatever process is represented by the incorporation, such process could take place four times in 24 hours. If this process is protein synthesis, it would require close to 720 Kcal.

Even though exact data are not available concerning the size of the various pools in which the label undergoes dilution, it is most clear

that many times proteins need to be "synthesized" and subsequently "broken down" before the label reaches its maximum concentration in the reaction products (Proteins). The "absolute rate of exchange," which represents the absolute amount of labelled plus non-labelled amino acids incorporated into protein per unit of time is not easy to calculate. Several mathematical treatments have been published, all of which refer only to very simple systems (234). It is nevertheless not difficult to see that, taking all the conditions mentioned above to hold true, the total supply of energy available to the individual (on the average about 3000 Kcal. per day) could be exceeded. In addition, such biological work as the evaporation of close to one liter of water by perspiratio insensibilis (worth about 500 Kcal.) the osmotic work, the mechanical work and thermoregulation, to mention only a few, would remain to be accounted for energy-wise.

The second problem is a kinetic one. If we would calculate the rate of protein synthesis, even assuming idealized molecules, (e.g. all having the same arbitrary molecular weight and composed of amino acids in one-to-one ratios), due to the loss of an elevated number of rotational and translational degree of freedom and the very significant increase in entropy which takes place when peptide bonds are formed, we would obtain, according to the classical theory, results of such a nature that the growth of even a simple dog would require eons.

This is possibly a more general problem, not limited to the biosynthesis of such polymers as proteins.

A "thermodynamics of irreversible processes" is developing to cope with problems which are beyond the reach of classical thermodynamics

(239). It is not to be excluded that a revision and extension into a more general formulation of some of the statistical concepts on which chemical kinetics is based, would be in order to explain biological mechanisms.

Ergonic Regulation of Growth

One of the most characteristic properties of living matter seems to be its ability to regulate its own growth, its ability to reach and maintain a determinate size, which becomes one of the distinctive attributions of that species.

Not all the factors involved in such a function are known, and their mechanism of action is still largely a mystery. A certain amount of speculation is thus inevitable in dealing with this subject. It appears legitimate, nevertheless, to classify growth regulators as "Intracellular" and "Extracellular," the former group including all factors which exert their function within the cell by which they have been produced, the latter group including all the others. The existence of intracellular growth factors has been postulated to explain specific behavior of cells, but the search for their isolation and identification has been unsuccessful. Recently, Holland and his group (240-241) have described a dialyzable factor, isolated from the tumor itself, and capable of inhibiting the growth of the tumor from which it has been isolated. This observation, when confirmed and extended, could represent the first solid ground on which a whole new chapter of biology may develop.

The ability to regulate growth has been demonstrated for a number

of hormones. The practically unlimited literature on this field has been recently reviewed (242). It is beyond our scope to give a complete analysis of this subject; the reader is thus referred to the quoted article by Wilhelmi and Russell. More specific information concerning the physiological properties and chemical constitution of single hormones can be found in more specialized papers (243-250). Our discussion will be limited to the protein anabolic properties of androgens.

Our knowledge concerning the influence of androgens on nitrogen metabolism has widely developed since the first observations by Kochakian and by Kochakian and Murlin (251) in 1935. These authors have shown that "male hormone" extracts from the urines of healthy medical students, prepared according to the method of Funk and Harrow (252) and tested for their androgenic properties by the usual bioassay methods, induced, when injected into castrated dogs, an unquestionable nitrogen retention. The retention was accounted for almost completely by a decrease in the urea-nitrogen output, and was elicited either by a single large dose of extract or by repeated small doses. An interesting fact was that increased dosage or prolonged treatment did not modify the amount of nitrogen retained. The "maximum" nitrogen retention was found to lie between 50 and 60 mg. per Kg. body weight per day. The nitrogen retention was paralleled by a corresponding increase in body weight.

Discontinuation of treatment resulted in the elimination of some "extra" nitrogen, a phenomenon called by Kochakian "the rebound effect."

In these experiments only two dogs were used, a "fat" dog and a "thin" one. Whereas no substantial differences existed between the nitrogen retention elicited in the two dogs by the administration of the urine

extract, the "rebound" was seen consistently on the "fat" dog but only in one experiment in the "thin" dog. The amount of nitrogen lost as a consequence of the rebound was only a fraction of that retained during the treatment, and the possibility that the "rebound" represented "labile" nitrogen, not yet incorporated into stable tissue structures has been discussed (253-254). More recently (255), data have been published pointing out a "labile" fraction contained in the liver, which is rapidly cleaved in the early phases of fasting and could represent an intermediate phase between food nitrogen and stable structure.

The relationship between androgen-induced nitrogen retention and excess nitrogen in the diet were studied and found largely independent (254).

These first observations have been extended by further studies by Kenyon and his group (256) and by McCullagh and Rossmiller (257) in man. Recent experiments in Kochakian's laboratory have confirmed again the nitrogen-retaining properties of androgens (testosterone propionate was used in this latter study), and again have pointed out that the total amount of nitrogen retained is a constant characteristic of any given animal, and is independent of the length of treatment (258-259).

The synthesis of androsterone (260), testosterone (261) and androstenedione (262), and the progressive elucidation of the endocrine function of the testes prompted Kochakian to study the anabolic properties of these compounds (253-254). When these hormones were used, a retention was demonstrated fully comparable qualitatively to the one shown with the urine extract. It was noted nevertheless (263) that none of the crystalline steroids was comparable in potency to the old urinary

extract. The eventual presence in the urines of another hormone, particularly active as an anabolizer had remained unsolved.

The experiments done on dogs were soon extended to rats, with analogous results (263). Similar retentions were obtained with various dose levels, included between 1.0 mg. and 7.5 mg. testosterone propionate per day, and with various diets, some of which contained up to 30 per cent protein while some were fortified with cystine, again confirming the relative independence of the total nitrogen retained from either dose regime or protein intake.

The extensive studies with androgens in man, performed by Kenyon and his group, were confirmed by Bassett, Keutmann and Kochakian (264-271). The authors have injected a 21 year old eunuchoid male with 25 mg. per day testosterone propionate and observed a marked retention, whose maximum per day was not reached until the sixth day. This certainly represents a somewhat delayed action, compared to the dogs, which reached their maximum on the average, on the third. This, and the fact that rats (263) retained a quantity about five times as great as dogs, per unit weight, but with the same "rebound effect" and with the same independence from the total doses received, would point out only quantitative differences in the response of various species to the administration of testosterone.

The nitrogen-retaining properties of androgens were further demonstrated in the depancreatized dog (272), as well as in the dog depancreatized and castrated (258-259). The use of N^{15} - labelled glycine has again confirmed their properties (273-274).

In an effort to elucidate the mechanism of action of androgens,

a long and exhaustive series of studies have been conducted in Kochakian's laboratory in the last fifteen years. Thus, the same author and his group (263) have shown that testosterone propionate produced an increase in arginase activity of the kidneys (but not of the livers) of rats, which continued even after the nitrogen-retaining effect has "worn off." In liver of normal and diabetic rats, a high protein diet (70 per cent casein) was able to produce increased arginase activity, which per se was insensitive to stimulation by testosterone. A small concentration of testosterone was also able to stimulate respiration of kidney slices in vitro. At high concentrations of testosterone, the opposite effect was shown.

Continuing along the same line, Endahl and Kochakian (275) have found that D-aminoacidoxidase content in the kidney of the castrated mouse is increased by administration of a wide variety of androgens. This increase was proportional to the increase in organ weight, so that it is difficult to say whether it is a cause rather than an effect of the increased size. In castrated rats and guinea pigs both weight and enzyme content of liver and kidney were not increased by the androgen administration. Analogous experiments conducted by Van Bekkum and Kassenaar (276) gave results in agreement with the ones obtained by Kochakian.

Also, Kochakian and Endahl have shown that a number of androgens increase, in the castrated guinea pig, both weight and transaminase activity of temporal muscle, being without effect on kidney, heart or liver (277). The species specificity of some of the testosterone-induced effects is demonstrated by the fact that in the castrate-thyroidectomized rat the weight of heart and kidney is reduced. Testosterone administration

repairs this defect in the castrate but not in the thyroidectomized animal. This observation shows species difference and points out the complex problem of hormone interrelations.

The effect of testosterone on the temporal muscle of guinea pigs (278) prompted Kochakian and his group to study extensively this phenomenon. Thus, it was shown (279) that the changes in the internal organs and in 29 different skeletal muscles in the castrated rat were all in proportion to the change in body weight. This need not be so in all species as has been shown by various authors (280-281). Kochakian, et al. (282-283) have shown extensively that castration specifically decreases and administration of testosterone specifically increases the weight of particular sections of the musculature. This effect, particularly evident in temporal muscle and in masseter, was localized on the muscles of the head and neck, and to a lesser extent, to those of the shoulder and upper back. Also (284-285), the same authors have shown that administration of testosterone specifically protects, in the guinea pig, the same muscles from weight loss after prolonged fasting.

It has been suggested (286) that the myotrophic effect of testosterone takes place only in those muscles which are important to the sexual activity of the male of a particular species. It is nevertheless unquestionable that, e.g. the temporal muscle of guinea pig, represents an organ which decreases its protein content under well defined and easily reproducible experimental conditions (castration), and increases very significantly its nitrogen content under equally well defined and easily reproducible conditions such as the administration of a crystalline, perfectly well known substance. It should thus represent an ideal system

to study protein synthesis, both *in vivo* and *in vitro*.

The possibility of satisfying easily and at will Gale's condition 1 and 2 in the same animal allows for flexibility, and persuaded us to study the effect of testosterone propionate on the incorporation of C^{14}-Glycine.

CHAPTER II

EXPERIMENTAL PROCEDURE

The incorporation of glycine 2-C^{14} into guinea pig proteins in normal animals and in animals previously castrated and treated with testosterone propionate is studied in the present series of experiments. The various steps of our procedure will be presented as separate sections: This will offer us the opportunity for a brief stepwise criticism of analogous methods of procedure and for discussion of their validity and limitations.

Preparation of Protein Powder, Plating and Counting

Several methods are available for the preparation of "protein powders" (287-289). They are all capable of yielding a substance free from soluble nitrogenous compounds, nucleic acids, fats and nitrogenous compounds linked to the main chain through bonds other than peptide bond (e.g. disulfide bridges). We have followed the one described by Peterson and Greenberg (289), which we shall illustrate briefly in its adaptations to our particular needs.

About 0.5 grams of tissue were homogenized with a Potter glass homogenizer in 10 ml. of cold 10 per cent trichloroacetic acid (TCA). The precipitate separated by centrifugation for 15' at 3000 rpm was resuspended in 5 ml. of 5 per cent TCA, and heated for 15' at 90°C. in order

to remove nucleic acid (290). This procedure, with the omission of the heating, was repeated three times giving a total of five precipitations in cold TCA. Further washing was represented by 5 ml. of ethyl alcohol (once) 5 ml. of a 3:1 alcohol-ether mixture (three times) and finally 5 ml. of dry ether (once). During the first wash with the alcohol-ether mixture, the suspension was heated for 3' at 60° C. The resulting powder was then stored in a desiccator to await counting.

All the supernatants were pooled in two main fractions (the TCA fraction and the alcohol-ether) and concentrated about 10 times by lyophylization.

We found it very important to use absolutely dry ether (Mallinkrodt). Even traces of moisture in the final ether wash prevented successful plating.

The effect of successive TCA washings on the presence of nitrogen in the supernatant was tested as follows: 1.656 grams of guinea pig muscle were homogenized with 15 ml. of cold 10 per cent TCA and the precipitate resuspended four times in 15 ml. of 10 per cent TCA. Nitrogen was determined according to the Micro-Kjeldahl method as currently used in the laboratory (282). Table 3 shows that repeated washings do not extract nitrogen from the precipitate.

The possibility that some of the label could be incorporated by a disulfide linkage was evaluated comparing the activity of powders before and after mercaptoethanol wash (289). It was found that practically 100 per cent of the label was mercaptoethanol-resistant, and we omitted this step from the procedure.

The nitrogen content of our powders was tested, to be used as a parameter of their "purity" as protein. It was found that they contained

15.4 per cent nitrogen, and this satisfied us that no appreciable quantities of extraneous materials were present.

TABLE 3

EFFECT OF REPEATED TCA WASHINGS ON THE CONCENTRATION OF NITROGEN IN THE SUPERNATANT

Wash #	Nitrogen (mg.)	% Total N
1	6.41	8.84
2	0.43	0.59
3	0.13	0.18
4	0.10	0.15
5	0.00	0.00

An aliquot of the dry sample was then used for plating. A planchet (as purchased from Nuclear-Chicago) was interposed between the screw-cap and the mouth of a porcelain jar, which had previously been cut at about 1 1/4 in. from its mouth. The powder aliquot was then suspended in 5 ml. dry ether by gentle homogenization, and the suspension poured into the jar. The ether was then evaporated at room temperature and the plated powder recovered after removal of the jar cap. The powder was evenly distributed on a circular area of 2.2 cm. diameter. Since the plating area was kept constant for all samples, we find it easier to speak in terms of the sample weight instead of its thickness.

We found that the best plating could be obtained, in our

conditions, with samples of about 10 ± 3 mg. in weight, smaller samples being less accurate due to the difficulty of weighing and recovering very small quantities of powder, thicker samples being less satisfactory due to the tendency of the more superficial layers to slough off when e.g. 15-20 mg. samples were prepared. Liquid samples (TCA fractions, etc.) were plated on stainless steel cups purchased from "Nuclear-Chicago," evaporated to dryness and counted.

Counting was performed using a Nuclear-Chicago scaler (Model 183B) connected to an automatic sample-changer (Model C-110B) and to a printing timer (Model C111B) from the same manufacturer. We used both gas-flow counters and regular G.M. tubes with a particularly thin mica window (1.2 mg/cm^2). The gas-flow counter permitted an efficiency: $\frac{\text{(Counts registered - BKG)}}{\text{Disintegration/min.}}$ of up to 35% (with the "Micromil" window in place) but this advantage was offset by the variability of the "plateau" slope, which in our apparatus oscillated between 4 and 7 per cent over a 200 volt range. We, thus, found more satisfactory the use of a mica-end regular G.M. tube, associated with either the use of larger labelling doses or concentration of the samples (in the case of liquid samples). The relatively low (12%) efficiency of such tubes was amply compensated by a very good stability of the plateau.

Duplicate samples were prepared and each sample was counted three times to a preset count of 12800 counts, which affords a standard error on every run of less than 1 per cent. The standard error of the average (over each count in each of the two samples) was thus of the order of magnitude of 0.3 per cent, thus setting the limit of acceptability for duplicate samples.

The problem of self-absorption was solved as follows: Since we could not use "infinitely thick" samples for the reasons outlined above, samples of weight ranging between 5 mg. and 20 mg. were prepared according to the procedure outlined in the first part of this section. The counts per minute and per milligram were then plotted versus sample weight and the hypothetical count at 0 thickness was extrapolated. The results were afterward recalculated, in order to express them as a per cent of the count at zero thickness. A logarithmic relationship was found between counts/milligram and weight.

Since 10 $\pm$ 3 mg. afforded the best samples, as far as handling ease was concerned, the quantity C_o^{10} was used to express our data relative to powders. It represents the counts relative to 10 mg. of powder, extrapolated to 0 thickness. From the data shown in Table 4, we found that:

$$C_o^{10} = \frac{10^3 C_W}{W \times \text{Exp.}_{10} (2 - 1.12 \times 10^{-2W})}$$

where C_W represents the counts/minute of the sample of weight W. Accordingly, we found that the self-absorption of a 10 mg. sample is 23 per cent of its C_o^{10}.

TABLE 4

SELF-ABSORPTION CURVE

Sample W	Counts	Counts/mgm.	Log counts/mgm.
5	463	93.0	1.96848
10	815	81.5	1.91116
15	1080	72.0	1.85733
20	1242	62.0	1.79239

Incorporation of C^{14} into Various Tissue as a Function of Time

Different organs and tissues incorporate labelled material at very different rates (197), making the choice of the time span between the injection of C^{14} AAc and sacrifice of the animal of primary importance.

In order to have a more adequate idea of the dynamism of C^{14} in the guinea pig, several animals were injected with 25 uC C^{14} aspartic acid and killed at various time intervals by skull fracture followed by exsanguination. Blood, kidney muscle and liver were removed and counted according to the procedure outlined in section IIA. The results are presented in Figure 1. Our results are in good agreement with those in the literature (289). A 12-hour lapse between injection and sacrifice was chosen as the most satisfactory.

In pilot experiments also a suitable dose of C^{14} was sought. C^{14}-aspartic and C^{14}-glycine were injected. The incorporation of C^{14}-aspartic acid was found to be only about 10 per cent of the incorporation achieved with C^{14}-glycine. This latter AAc was chosen for further experimentation.

A dose of 50 uC per guinea pig appeared to be quite satisfactory, giving suitable counting also in organs like muscle, whose protein turnover is generally accepted as being low.

Since we were planning to dissect a relatively large number of organs from the same animal, the question arose of what the maximum time after death of the animal would be, in which organs could be removed, before autolysis phenomena would interfere. In order to clarify this point, one guinea pig was injected and muscles removed at different time intervals after death of animal, starting with a first group dissected 45' after death and ending with a last group dissected at 170'. Results, as

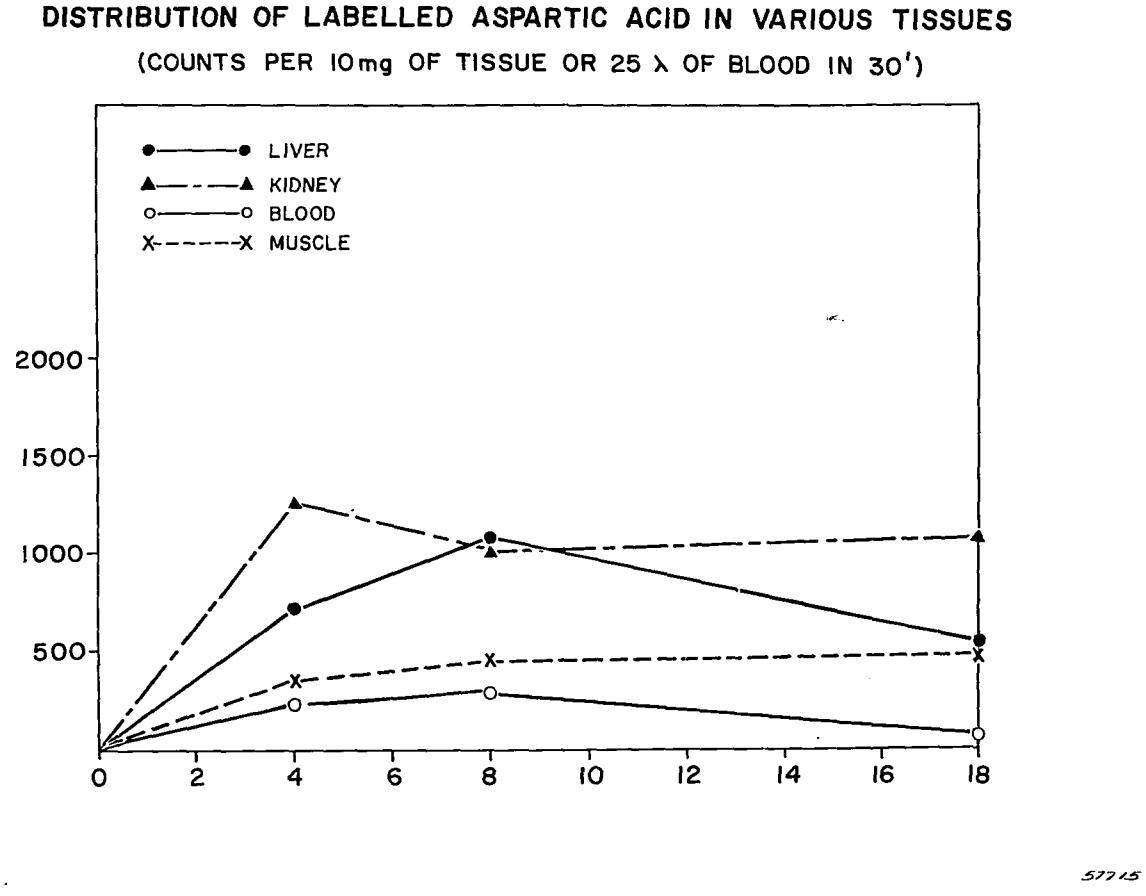

Figure 1. Distribution of Labelled Aspartic Acid in Various Tissues.

shown in Table 5 indicate no significant decrease in label content during the time tested.

Effect of Castration and Testosterone Propionate Treatment on Weight and Glycine-2-C^{14} Incorporation

Male guinea pigs from the Zeimet Bio-farms were used for the experiment and divided into three groups. Group I represented the control, while Groups II and III were castrated at the age of 68 days, when they weighed 549 to 698 grams. The animals were maintained in an air-conditioned room and fed Rockland Guinea Pig Diet supplemented with 30 g. of carrots and 25 mg. Vitamin C twice a week.

After castration, Group III received daily injections of 10 mg. testosterone propionate[1] dissolved in 0.2 ml. sesame oil, while Groups I and II received an equal volume of sesame oil. The injection period lasted three weeks, according to previous observations (291) indicating that the above treatment is the best suited to bring about maximum tissue stimulation. Body weight was determined three times a week, to ascertain a proper growth curve.

On the twenty-first day of treatment, animals were transferred to different cages and deprived of food. After 12 hours of fasting, 50 micro-Curies of glycine-2-C^{14} were injected intraperitoneally and the animals killed, following a second 12 hour period of fasting, by exsanguination. The organs were rapidly removed and weighed, and aliquots of about 500 mg. or less were dropped in 10 ml. of cold 10 per cent TCA, and then processed as specified in a previous section. The procedure

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[1]The androgen was provided as Perandren propionate by CIBA Pharmaceutical Products.

TABLE 5

C^{10}_{o} IN VARIOUS MUSCLES DISSECTED AT DIFFERENT TIMES AFTER DEATH OF THE ANIMAL

Group I (60')	Group II (75')	Group III (90')	Group IV (105')	Group V (120')	Group VI (125')	Group VII (150')	Group VIII (165')	Group IX (170')
480	495	470	533	511	560	120	570	542
260	414	476	577	513	520	300	550	530
500	522	483	162	450	460	514	595	508
531	468	580	172	480	550	550	595	-
484	376	-	484	460	480	310	-	-
-	477	-	296	-	530	190	-	-
-	-	-	-	-	-	524	-	-
-	-	-	-	-	-	524	-	-
Ave.451	458	502	371	483	520	379	579	516

involving dissecting and weighing required less than 120 minutes, thus being well within the safe period, as determined by the data presented in Table 5.

Average weight and S. E. of the various organs are shown in Table 6. The agreement with the data previously obtained (291) is quite satisfactory.

In Table 7, the average powder counts are presented. They are expressed as C^{10}_{o} and as counts per minute and per gram of fresh tissue. These latter values have been calculated from the known values for C^{10}_{o}, the weights of fresh samples and the powder yield.

The values for the radioactivity in the TCA supernatants, expressed as counts per minute and per gram of fresh tissue to be comparable with the analogous data for the powders, are shown in Table 8. Only negligible amounts of radioactivity were found in the "alcohol-ether supernatant" and those data are thus omitted.

Figure 2 shows the average relative weights and counts for those organs in which any significant change, be it in weight or counts, has been demonstrated. The values for the normals have been arbitrarily taken equal to 100, and proportional values for the other groups have been calculated. The weights of seminal vesicles and prostate are presented together, due to the difficulty in separating them satisfactorily, while separate counts for the two organs are given.

As it can easily be seen, no data appear in this figure concerning the TCA supernatants. Their large standard errors prevented drawing of useful conclusions in every case, except in the case of seminal vesicles, whose behavior followed closely the pattern presented by the

TABLE 6

EFFECT OF CASTRATION AND OF TESTOSTERONE PROPIONATE ON THE WEIGHT OF SEVERAL ORGANS AND MUSCLES OF GUINEA PIG

Organ	GROUP I Normal Weight (mgm.)	GROUP II Castrated Weight (mgm.)	GROUP III Castrated and T. P. Weight (mgm.)
Sex Tissue			
Retractor Penis	126 ± 6	57 ± 5	111 ± 8
Head and Neck			
Digastric	524 ± 53	348 ± 20	482 ± 11
Masseter	2042 ± 80	1529 ± 90	2405 ± 155
Temporal	964 ± 41	477 ± 19	862 ± 50
Clavotrapezius	429 ± 18	423 ± 29	486 ± 12
Levator scapulae	632 ± 15	533 ± 36	654 ± 33
Sternomastoid	245 ± 57	133 ± 7	174 ± 20
Chest			
Pectorals	2337 ± 47	2150 ± 108	2424 ± 89
Serratus Anterior	1318 ± 93	1155 ± 41	1291 ± 89

TABLE 6-Continued

Organ	GROUP I Normal Weight (mgm.)	GROUP II Castrated Weight (mgm.)	GROUP III Castrated and T. P. Weight (mgm.)
Shoulder and Back			
Rhomboideus	1147 ± 59	977 ± 43	1012 ± 155
Acromiotrapezius	901 ± 53	695 ± 37	912 ± 73
Spinotrapezius	675 ± 55	577 ± 23	877 ± 98
Latissimus dorsi	1751 ± 225	1635 ± 157	1945 ± 77
Body Wall and Spine			
Diaphragm	2659 ± 118	2698 ± 114	2807 ± 95
Internal and External Obliques	9580 ± 920	8756 ± 408	9434 ± 666
Quadratus Lumborum	6040 ± 241	5764 ± 273	6038 ± 111
Psoas minor	1062 ± 20	1032 ± 67	1217 ± 97
Psoas major	1761 ± 165	1329 ± 65	1603 ± 62
Scapula			
Subscapularis	1192 ± 74	1021 ± 27	1162 ± 24

TABLE 6-Continued

Organ	GROUP I Normal Weight (mgm.)	GROUP II Castrated Weight (mgm.)	GROUP III Castrated and T. P. Weight (mgm.)
Teres major	901 ± 61	787 ± 35	908 ± 29
Infraspinatus	1281 ± 51	1144 ± 20	1445 ± 37
Supraspinatus	1611 ± 136	1412 ± 92	1664 ± 57
Spinodeltoid	695 ± 61	608 ± 50	671 ± 27
Acromoideltoid	337 ± 31	294 ± 20	320 ± 14
Forearm			
Radial-ulnar complex	1517 ± 75	1454 ± 23	1550 ± 94
Clavobrachialis	259 ± 10	214 ± 10	250 ± 21
Biceps brachii	442 ± 32	406 ± 22	415 ± 12
Brachialis	414 ± 23	393 ± 14	417 ± 12
Lateral head of triceps	525 ± 38	419 ± 13	475 ± 14
Long head of triceps	2197 ± 66	2054 ± 31	2264 ± 96
Hip			
Gluteus medius	4932 ± 205	4332 ± 162	5123 ± 261

TABLE 6-Continued

Organ	GROUP I Normal Weight (mgm.)	GROUP II Castrated Weight (mgm.)	GROUP III Castrated and T. P. Weight (mgm.)
Gluteus maximus	836 ± 13	786 ± 26	906 ± 55
Caudofemoralis	322 ± 15	355 ± 25	387 ± 35
Semimembranosus	1738 ± 62	1534 ± 32	1801 ± 87
Semitendinosus	2007 ± 57	1663 ± 38	2109 ± 69
Biceps femoris	4871 ± 133	4414 ± 93	4895 ± 39
Thigh			
Rectus femoris	1938 ± 64	1823 ± 37	1851 ± 111
Vastus lateralis	1835 ± 98	1616 ± 24	1832 ± 119
Pectineus	332 ± 29	309 ± 10	311 ± 19
Adductor longus	214 ± 8	199 ± 7	238 ± 18
Adductor brevis	736 ± 34	670 ± 21	724 ± 49
Gracilis	1578 ± 103	1395 ± 23	1513 ± 102
Shank			
Tibialis posterior	381 ± 18	356 ± 21	395 ± 45

TABLE 6-Continued

Organ	GROUP I Normal Weight (mgm.)	GROUP II Castrated Weight (mgm.)	GROUP III Castrated and T. P. Weight (mgm.)
Peroneus	468 ± 14	466 ± 10	440 ± 26
Flexor hallicus longus	588 ± 35	552 ± 11	647 ± 41
Tibialis anterior	729 ± 10	694 ± 17	703 ± 25
Soleus	200 ± 22	201 ± 7	259 ± 31
Gastrocnemius	2114 ± 122	1891 ± 40	2161 ± 135
Organ Weights			
Liver	21670 ± 3000	21667 ± 2307	20558 ± 1092
Kidney	3942 ± 17	4102 ± 107	4457 ± 180
Heart	1519 ± 73	1380 ± 135	1545 ± 77
Spleen	701 ± 43	691 ± 45	695 ± 57
Adrenal	309 ± 26	367 ± 6	262 ± 12
S.V. and Pr	3788 ± 200	940 ± 34	3757 ± 250
Urinary Bladder	429 ± 14	372 ± 27	459 ± 25
Thymus	869 ± 116	1130 ± 316	1275 ± 57

TABLE 7

INCORPORATION OF GLYCINE-2-C^{14} INTO PROTEINS OF VARIOUS GUINEA PIG ORGANS

Organ	Group I (Control)		Group II (Castrated)		Group III (Castrated and T. P.)	
	cpm/10 mg.	cpm/gm.wet wt.	cpm/10 mg.	cpm/gm.wet wt.	cpm/10 mg.	cpm/gm.wet wt.
Sex Tissue						
Retractor Penis	97 ± 6	1986 ± 129	90 ± 13	1827 ± 314	183 ± 12	3703 ± 209
Head and Neck						
Digastric	186 ± 17	3319 ± 277	185 ± 14	3302 ± 268	189 ± 16	3360 ± 277
Masseter	198 ± 12	3899 ± 212	180 ± 9	3556 ± 221	223 ± 14	4459 ± 354
Temporal	99 ± 8	1979 ± 170	93 ± 3	1832 ± 64	133 ± 8	2754 ± 180
Clavotrapezius	144 ± 14	2517 ± 191	129 ± 5	2150 ± 101	175 ± 17	2920 ± 270
Levator scapulae	148 ± 16	2613 ± 258	118 ± 9	2063 ± 165	155 ± 22	2647 ± 369
Sternomastoid	147 ± 26	2857 ± 506	158 ± 12	3158 ± 205	187 ± 20	3505 ± 407
Chest						
Pectorals	134 ± 14	2777 ± 308	134 ± 12	2702 ± 272	149 ± 17	3020 ± 342
Serratus Anterior	159 ± 19	2936 ± 373	119 ± 9	2241 ± 149	146 ± 19	2688 ± 342

TABLE 7-Continued

Organ	Group I (Control)		Group II (Castrated)		Group III (Castrated and T. P.)	
	cpm/10 mg.	cpm/gm.wet wt.	cpm/10 mg.	cpm/gm.wet wt.	cpm/10 mg.	cpm/gm.wet wt.
Shoulder and Back						
Rhomboideus	149 ± 11	2586 ± 180	145 ± 18	2519 ± 306	166 ± 15	2878 ± 235
Acromiotrapezius	127 ± 26	2103 ± 426	115 ± 7	1910 ± 97	153 ± 15	2564 ± 254
Spinotrapezius	174 ± 9	2632 ± 153	165 ± 13	2468 ± 183	190 ± 28	2823 ± 383
Latissimus dorsi	142 ± 17	2664 ± 328	163 ± 27	3062 ± 494	164 ± 19	3072 ± 363
Body Wall and Spine						
Diaphragm	338 ± 11	5307 ± 140	235 ± 12	3675 ± 196	344 ± 13	5435 ± 187
Internal and External Obliques	327 ± 38	6253 ± 721	283 ± 30	5565 ± 596	497 ± 49	9448 ± 1630
Quadratus Lumborum	109 ± 16	2253 ± 287	111 ± 6	2305 ± 125	97 ± 15	2003 ± 209
Psoas minor	135 ± 8	2125 ± 123	142 ± 10	2248 ± 159	159 ± 16	2509 ± 233
Psoas major	135 ± 13	2353 ± 245	131 ± 7	2070 ± 88	143 ± 15	2456 ± 268
Scapula						
Subscapularis	194 ± 4	3419 ± 79	174 ± 17	3015 ± 260	186 ± 16	3216 ± 260
Teres major	162 ± 16	3568 ± 371	146 ± 11	3108 ± 234	147 ± 4	3172 ± 72

TABLE 7-Continued

Organ	Group I (Control)		Group II (Castrated)		Group III (Castrated and T. P.)	
	cpm/10 mg.	cpm/gm.wet wt.	cpm/10 mg.	cpm/gm.wet wt.	cpm/10 mg.	cpm/gm.wet wt.
Infraspinatus	183 ± 18	3761 ± 346	163 ± 13	3390 ± 271	180 ± 17	3682 ± 330
Supraspinatus	162 ± 13	3208 ± 112	162 ± 9	3238 ± 194	220 ± 20	4495 ± 442
Spinodeltoid	140 ± 16	2858 ± 101	126 ± 8	2564 ± 190	158 ± 21	3201 ± 401
Acromiodeltoid	154 ± 10	2979 ± 172	129 ± 11	2463 ± 190	156 ± 25	3022 ± 471
Forearm						
Radial-ulnar complex	168 ± 9	3076 ± 162	175 ± 11	3207 ± 276	178 ± 11	3210 ± 149
Clavobrachialis	135 ± 12	2756 ± 246	113 ± 8	2324 ± 139	146 ± 9	3018 ± 164
Biceps brachii	169 ± 10	3249 ± 220	178 ± 38	3457 ± 752	166 ± 16	3274 ± 284
Brachialis	168 ± 15	3355 ± 310	162 ± 10	3217 ± 177	166 ± 27	3315 ± 524
Lateral head of triceps	154 ± 12	3104 ± 240	158 ± 8	2974 ± 432	196 ± 27	3899 ± 530
Long head of triceps	125 ± 14	2857 ± 85	107 ± 11	2407 ± 229	137 ± 16	3151 ± 340
Hip						
Gluteus medius	105 ± 7	2057 ± 117	104 ± 19	2044 ± 320	107 ± 15	2057 ± 290
Gluteus maximus	128 ± 10	2135 ± 147	141 ± 15	2342 ± 266	136 ± 16	2255 ± 262

TABLE 7-Continued

Organ	Group I (Control)		Group II (Castrated)		Group III (Castrated and T. P.)	
	cpm/10 mg.	cpm/gm.wet wt.	cpm/10 mg.	cpm/gm.wet wt.	cpm/10 mg.	cpm/gm.wet wt.
Caudofemoralis	119 ± 9	1892 ± 143	112 ± 6	1812 ± 120	131 ± 15	2062 ± 220
Semimembranosus	148 ± 17	1947 ± 245	115 ± 6	1617 ± 90	139 ± 19	1947 ± 232
Semitendinosus	115 ± 12	1824 ± 271	136 ± 13	1924 ± 187	116 ± 15	1622 ± 220
Biceps femoris	128 ± 9	2442 ± 148	101 ± 11	1939 ± 209	112 ± 21	2133 ± 405
Thigh						
Rectus femoris	130 ± 21	2430 ± 389	131 ± 4	2391 ± 74	134 ± 13	2535 ± 265
Vastus lateralis	150 ± 12	2904 ± 226	109 ± 13	2093 ± 278	144 ± 15	2710 ± 272
Pectineous	143 ± 13	2637 ± 255	117 ± 9	2281 ± 159	121 ± 18	2606 ± 343
Adductor longus	247 ± 21	3989 ± 285	235 ± 9	3839 ± 179	198 ± 8	3200 ± 75
Adductor brevis	138 ± 17	2805 ± 108	129 ± 11	2525 ± 196	150 ± 22	3026 ± 419
Gracilis	124 ± 19	2069 ± 314	105 ± 9	1719 ± 164	111 ± 22	1800 ± 357
Shank						
Tibialis posterior	184 ± 8	3315 ± 237	173 ± 13	3330 ± 211	188 ± 15	3619 ± 297
Peroneus	159 ± 12	2689 ± 177	162 ± 11	2724 ± 169	160 ± 15	2780 ± 232

TABLE 7-Continued

Organ	Group I (Control)		Group II (Castrated)		Group III (Castrated and T. P.)	
	cpm/10 mg.	cpm/gm.wet wt.	cpm/10 mg.	cpm/gm.wet wt.	cpm/10 mg.	cpm/gm.wet wt.
Flexor hallicus longus	125 ± 16	2188 ± 328	128 ± 1	2349 ± 46	128 ± 16	2172 ± 270
Tibialis anterior	169 ± 21	3116 ± 403	156 ± 22	2765 ± 384	168 ± 28	3065 ± 517
Soleus	206 ± 17	3413 ± 256	212 ± 13	3517 ± 191	229 ± 36	3617 ± 552
Gastrocnemius	130 ± 17	1999 ± 107	132 ± 11	2054 ± 181	141 ± 16	2172 ± 245
Organs						
Liver	670 ± 20	14038 ± 496	605 ± 11	12639 ± 239	607 ± 11	12722 ± 235
Kidney	621 ± 28	10056 ± 41	562 ± 27	9114 ± 443	608 ± 14	10185 ± 317
Heart	401 ± 38	7057 ± 667	421 ± 41	7378 ± 658	421 ± 44	7353 ± 767
Spleen	553 ± 17	9068 ± 213	617 ± 19	9473 ± 980	602 ± 29	9917 ± 520
S. V.	592 ± 82	8434 ± 1171	383 ± 53	5460 ± 757	970 ± 108	13705 ± 1519
Prostate	233 ± 12	3630 ± 220	192 ± 23	2115 ± 363	393 ± 44	6081 ± 714
Stomach	953 ± 26	13100 ± 357	925 ± 81	12312 ± 956	1000 ± 76	13660 ± 1042
Intestine	978 ± 19	13182 ± 260	981 ± 43	13125 ± 573	985 ± 47	13002 ± 620
Urinary Bladder	725 ± 129	10282 ± 1811	849 ± 189	12151 ± 2670	698 ± 106	9833 ± 1542

TABLE 8

DISTRIBUTION OF GLYCINE 2-C^{14} IN THE TCA SUPERNATANT OF VARIOUS GUINEA PIG ORGANS
(Counts/minute/g.wet wt.)

Organ	GROUP I Normal	GROUP II Castrated	GROUP III Castrated and T. P.
Sex Tissue			
Retractor Penis	2118 ± 132	1932 ± 120	2615 ± 215
Head and Neck			
Digastric	2212 ± 134	1873 ± 36	1975 ± 379
Masseter	1932 ± 112	2016 ± 147	1943 ± 369
Temporal	2010 ± 477	1282 ± 166	1644 ± 395
Clavotrapezius	2266 ± 168	1891 ± 109	2281 ± 108
Levator scapulae	1485 ± 204	2111 ± 353	1421 ± 456
Sternomastoid	1044 ± 513	1507 ± 161	1514 ± 274
Chest			
Pectoralis	2337 ± 190	2813 ± 556	2183 ± 383
Serratus anterior	1849 ± 320	1572 ± 195	2215 ± 273

TABLE 8-Continued

Organ	GROUP I Normal	GROUP II Castrated	GROUP III Castrated and T. P.
Shoulder and Back			
Rhomboideus	1785 ± 191	1912 ± 265	2340 ± 215
Acromiotrapezius	1990 ± 175	1811 ± 193	2003 ± 166
Spinotrapezius	1436 ± 152	1167 ± 247	1198 ± 129
Latissimus Dorsi	1744 ± 333	1995 ± 341	1525 ± 249
Body Wall and Spine			
Diaphragm	2372 ± 421	2107 ± 145	2223 ± 285
Internal and External Obliques	4815 ± 184	4105 ± 241	4917 ± 480
Quadratus Lumborum	2521 ± 446	2831 ± 443	1830 ± 750
Psoas minor	1728 ± 222	1523 ± 360	1216 ± 580
Psoas major	2097 ± 671	2512 ± 411	1464 ± 461
Scapula			
Subscapularis	2731 ± 360	2525 ± 379	1975 ± 415
Teres major	2187 ± 210	1793 ± 235	2215 ± 309

TABLE 8-Continued

Organ	GROUP I Normal	GROUP II Castrated	GROUP III Castrated and T. P.
Infraspinatus	1715 ± 410	1819 ± 218	1654 ± 179
Supraspinatus	1699 ± 311	2323 ± 265	2031 ± 247
Spinodeltoid	1383 ± 310	1450 ± 362	1982 ± 292
Acromiodeltoid	1507 ± 361	1099 ± 358	1548 ± 224
Forearm			
Radial ulnar complex	2817		
Clavobrachialis	2088 ± 347	1402 ± 119	1193 ± 230
Biceps brachii	2133 ± 283	1882 ± 199	1657 ± 312
Brachialis	1745 ± 345	1648 ± 228	1959 ± 149
Lateral head triceps	2156 ± 413	1929 ± 273	1931 ± 322
Long head triceps	2316 ± 193	1935 ± 210	1896 ± 333
Hip			
Gluteus medius	3080 ± 645	2148 ± 430	1798 ± 255
Gluteus maximus	2334 ± 466	2553 ± 120	1946 ± 549
Caudofemoralis	2191 ± 358	2189 ± 413	1710 ± 403

TABLE 8-Continued

Organ	GROUP I Normal	GROUP II Castrated	GROUP III Castrated and T. P.
Semimembranosus	2017 ± 275	2120 ± 243	1595 ± 619
Semitendinosus	2592 ± 502	2209 ± 384	1488 ± 516
Biceps femoris	2976 ± 545	3089 ± 528	2046 ± 501
Thigh			
Rectus femoris	1791 ± 452	1962 ± 264	1363 ± 467
Vastus lateralis	1499 ± 218	1951 ± 343	1259 ± 461
Pectineus	1544 ± 379	1438 ± 312	2367 ± 358
Adductor longus	2469 ± 204	2478 ± 158	1528 ± 296
Adductor brevis	1854 ± 312	1460 ± 336	1558 ± 493
Gracilis	2810 ± 484	2610 ± 455	2021 ± 543
Shank			
Tibialis posterior	1897 ± 414	1728 ± 374	1268 ± 361
Peroneus	1914 ± 409	1587 ± 105	1452 ± 244
Flexor hallicus longus	2185 ± 346	2270 ± 261	1857 ± 827

TABLE 8-Continued

Organ	GROUP I Normal	GROUP II Castrated	GROUP III Castrated and T. P.
Tibialis anterior	1588 ± 282	1878 ± 292	1283 ± 227
Soleus	1278 ± 138	859 ± 338	1388 ± 256
Gastrocnemius	2059 ± 279	2258 ± 513	1225 ± 382
Organs			
Liver	12679 ± 1039	14192 ± 4028	10764 ± 2271
Kidney	7308 ± 424	7467 ± 426	6162 ± 851
Heart	3384 ± 132	3094 ± 210	3642 ± 185
Spleen	6782 ± 681	7945 ± 498	7053 ± 873
S. V.	5275 ± 442	3258 ± 787	7985 ± 1231
Prostate	3058 ± 234	2393 ± 325	3718 ± 291
Stomach	8485 ± 411	7756 ± 889	6165 ± 231
Intestine	7265 ± 183	7934 ± 1766	6364 ± 1183
Urinary Bladder	4465 ± 215	3949 ± 223	4146 ± 382

corresponding powders.

It can be seen from the analysis of Table 6, 7, and 8 and from Figure 2 that the guinea pig organs respond to massive doses of testosterone in the classical manner so precisely described by Kochakian and his group (283). The response, particularly pronounced in the sex tissue, but also well evident in several of the head and neck muscles, is represented by decrease in weight as a result of castration and return to normal after testosterone treatment. It is noteworthy that massive doses of the steroid do not elicit a greater response than the one obtainable with relatively small doses. This fact has already been noted and commented upon.

Examination of Figure 2 indicates that increase in weight is paralleled, in every case except in the case of the digastric, by an increase of the incorporation of C^{14} per unit weight. It also shows that increased incorporation takes place, if we accept the behavior presented by the obliques, only in those organs which respond to stimulation with growth. A direct relationship seems thus to exist between the two phenomena. Some difference exists, nevertheless, between the characters of the two responses. While weight decreases after castration, to increase by testosterone treatment, castration has but little influence on the C^{14} uptake, which is stimulated only during the anabolic phase.

Several problems are posed by the results of the experiment discussed above.

The first one concerns the significance of the C^{14} uptake. From our data it can be seen that, although incorporation <u>per se</u> might be a parallel phenomenon only marginally related to protein synthesis, its

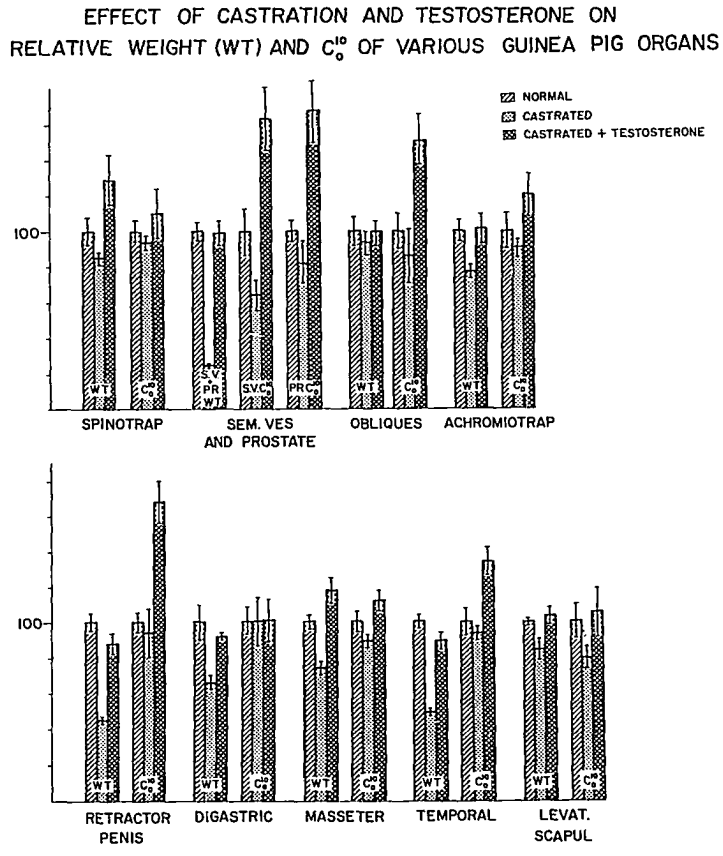

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Figure 2. Effect of Castration and Testosterone on Relative Weight and C_o^{10} of Various Guinea Pig Organs.

stimulation is possible only in those cases in which stimulation of growth is achieved. This condition is equivalent to Gale's condition 2. It is thus highly probable that incorporation and growth respond to the same stimuli in the same sense, thus making, in this particular case, the uptake a significant step in the synthesis of guinea pig protein.

It is interesting that an approximately equal amount of C^{14} is found in the supernatant and in the precipitate. Although very likely the former represents free amino acid, the most recent discovery by Gale (292) that a "TCA soluble intermediate" different from free amino acids represents probably one of the first steps in the builidng of proteins would make this fraction a very interesting hunting ground in the search for the reaction or reactions whose acceleration by testosterone is responsible for its anabolic properties. Unfortunately, the limited number of animals in our experiments prevented fruitful work in this direction.

The second problem is related to the nature of the "neoformed" protein and to its relationship with the so-called "normal structure and composition" of the organ. First of all it is of interest to know whether the grown organ is grown as result of hypertrophy or as a result of hyperplasia. It is not possible to arrive directly at a decision on the basis of our data. Experiments now in course in Dr. Kochakian's laboratory (293) would indicate that there is no increase in cell number after testosterone treatment. Photomicrographs of muscles show increase in sarcoplasm, thus characterizing the response to castration and testosterone treatment respectively, as depletion and replenishment of cytoplasmic protein.

These data, together with the now established fact that only part

of the guinea pig organs respond to testosterone injection raises the questions whether "all" cytoplasmic protein of the responsive organs respond or whether testosterone is capable of promoting synthesis of only a score of them. This second hypothesis would explain the difference in response between the various organs with the presence or absence of the "responsive protein."

To elucidate in part this interesting point we have undertaken fractionation of the protein powders.

Hydrolysis and Chromatography of Protein Powders

The study of the protein molecule depends in considerable part upon efficient methods for the separation of amino acids and or peptides present in hydrolysates. In general, the techniques that have proved most useful for these purposes are chromatography, liquid-liquid distribution and zone electrophoresis, used individually or in combination.

Paper chromatography, following its introduction in 1944 by Consden, Gordon and Martin (294), has become possibly the most familiar method of fractionation in protein chemistry and has played a central role in the acquisition of qualitative information in the field of amino-acids and peptides. This subject has been reviewed recently by Turba (295), by Lederer and Lederer (296), by Sanger (297), by Thompson and Thompson (298) and by Block, Durrum and Zweigh (299).

Paper chromatography of both TCA supernatant and protein hydrolysates was attempted with unsatisfactory results. The low concentration of C^{14}, on one hand, and the widely different concentration of the various AAc, on the other, did not allow a satisfactory, simultaneous

separation and quantitation of the AAc, and accurate measure of the radioactivity. We have thus returned to column chromatography which, even though it does not possess the speed and simplicity of chromatography on paper, does have the remarkable advantage that larger quantities of material can be used with satisfactory fractionation. For this purpose we have followed the technique described by Stein and Moore (300-302), which makes use of fine mesh resins prepared from styrene copolymerized with about 4 - 8 per cent divinylbenzene. In the following section, such a technique is described in detail.

About five pounds of resin (Dowex 50-X4 and Dowex 50-X5 obtained through the Dow Chemical Company, Midland, Michigan) were washed on large Buchner funnels with 4 N HCl until the effluent was colorless. This required between 4 and 8 liters of HCl per pound of resin. The bed was then washed free from acid with H_2O and washed with 2 N NaOH until the effluent was alkaline. The resin was then suspended in about three times its volume of 1 N NaOH and heated on a steam bath for three hours with frequent stirring. After settling for 1 hour, the supernatant was decanted out and the procedure repeated for a total of five times. The resulting sodium salt of the resin was then washed free from alkali and passed through a 400 mesh sieve. A good resin should yield about 50 - 60 per cent of "400 mesh preparation."

Chromatographic columns, obtained from Scientific Glass Apparatus Company, Bloomfield, New Jersey, were of the Zechmeister-Cholnoky type, with coarse sintered glass plates, and had an inner diameter of 0.9 cm. and a length of 165 cm. above the plate. The sintered plate was sealed directly into the tube: With this arrangement the ground joint at the

bottom of the tube (301) is eliminated, thus insuring a grease free tip and a more even flow of the effluent. Tubes were enclosed in two connecting West type condenser jackets (Kimble #18003-A and C) through which water was circulated from a constant temperature bath. Adequate temperature control ($\pm$ 1°) was satisfactorily obtained using a circulating pump (American Instrument Company #4-603-S) and an Aminco (#4-96) circulating water bath.

For the preparation of the columns, about 200 ml. of the settled sodium salt of the resin were suspended in an approximately equal volume of water and transferred on a Buchner funnel, where they were washed with 1 liter of 1 N NaOH, a filter paper on top of the resin bed being used to avoid channeling, and with the major part of 1 liter of 0.2 N buffer at pH 5 (1:10 dilution of the 2N buffer defined in Table 9). The resin was resuspended in the remaining buffer to give about equal volumes of settled bed and buffer. The suspension was freed from air bubbles by gentle stirring after the slurry had been allowed to stand for about two hours, and was then used for pouring columns. The original method suggested pouring in sections of about 20 - 30 cm. in height. We found it more practical to pour in sections each of which was equal to one half of the available length. The two methods of pouring were compared and found to yield substantially the same results. Each portion was poured through a funnel, the tip of which was maintained in contact with the wall of the column, and allowed to settle under a constant pressure of 15 cm. of Hg, (see below for the pressure regulating device,) until no further drop in height of the resin columns occurred. At that time, the supernatant buffer was withdrawn by suction through Tygon tubing (1/8 inch in outer

TABLE 9

REAGENTS REQUIRED FOR COLUMN CHROMATOGRAPHY OF AMINO ACIDS ACCORDING TO STEIN AND MOORE (301)

A. Buffers	Citric Acid·H_2O gm.	Acetic Acid (glacial) gm.	NaOH 97% gm.	Na Acetate 3 H_2O gm.	HCl conc. ml.	Final Volume l.
pH 2.2 ± 0.03* 0.2 N Na Citrate	105	---	42	----	80	5
pH 3.1 ± 0.03* 0.2 N Na Citrate	714	---	282	----	393	34
pH 5.1 ± 0.02** 0.0 N Na Citrate-Acetate	3570	730	1600	4630	---	34

* Before use, add 5 ml. BRIJ 35 solution (see below) and 5 ml. thiodiglycol (Carbide & Carbon Chemical Corporation, 30 East 42nd Street, New York 17, N. Y.) per liter.

** Before use, add 1 ml. BRIJ 35 solution and 5 ml. thiodiglycol per liter.

B. BRIJ 35. The solution is prepared by dissolving 50 gm. of BRIJ (Atlas Powder Co., Wilmington, Delaware) in 100 ml. hot water. If the detergent, a polyoxyethylene-lauryl-alcohol, does not remain fully in solution at room temperature, the amount of water should be increased to 150 ml., and 1.35 times the stated quantities of solution used.

diameter). The final column had a height of 150 cm. and exhibited a rate of flow of not less than 5 ml. per hour under 15 cm. pressure.

When the column was ready, it was connected through about three feet of Tygon tubing (B-44-3, 1/8 inch inner diameter) to a 1 liter bottle equipped with an outlet at the bottom. The connection between the Tygon tubing and the column was obtained through a glass U-tube passing through a micro rubber stopper (300). The upper mouth of the one liter bottle was stoppered with a #6 rubber stopper, through which another U-tube was passed, connecting with the pressure line.

A convenient volume of 0.2 N NaOH (containing 0.5 per cent BRIJ 35 solution. See Table 9) was placed in the one liter bottle and at least 150 ml. of that alkaline solution driven through overnight at a pressure of 15 cm. Hg.

A suitable regulation of the pressure exerted on the top of the columns was achieved in the following way. The laboratory compressed air was passed through a filter (The Moore Products Company, H & Lycoming Streets, Philadelphia, Pennsylvania, #2306) and a reducing valve (same manufacturer, #60) to reduce the pressure to 20 pounds/square inch, as indicated by a gauge (#1169-1). Air of the desired pressure was obtained from this line through the use of a Nullmatic pressure regulator (The Moore Products Company, #40-15) equipped with a 0 to 25 cm. Hg. outlet pressure gauge, obtained, upon kind suggestion from Doctors Stein and Moore, from J. Nageldinger & Son, Inc., 366 East 153rd Street, Bronx 55, New York. This gauge is somewhat different from the one originally described by Stein and Moore (301). In order to prevent clogging of the pressure-regulating system by rust, copper piping was used.

A day or so before the column was to be used, the NaOH solution above the resin, in the Tygon tubing and in the bottle was replaced by buffer (pH 3.1 for the powders. See Table 9). The buffer was driven through until the pH of the effluent had reached that of the influent. At that time, the flow was stopped. Further passage of buffer would have the effect of increasing the NH_3 blank for the column.

The buffers, as defined in Table 9, were prepared from reagent grade chemicals in lots of 17 liters and stored at 4° C. in Pyrex jars. If pH adjustment were necessary, it could have been done by utilization of the fact that, for the pH 3.1 buffer, 5 ml. of concentrated HCl corresponded to about 0.03 pH units, while for the pH 5.1 buffer 70 ml. of glacial acetic acid or 40 gm. NaOH did correspond also to 0.03 pH units.

In preparing the pH 5.1 buffer, citric acid was first dissolved in 15 liters and NaOH added in small portions, to avoid overheating.

The samples for chromatography were prepared as follows: 5 mg. of powder from each of the animals belonging to the same group were transferred to a 5 ml. ampoule. Two ml. 6 N HCl were added, the ampoule sealed and the sample hydrolyzed in an oven at 105° C. for 20 hours. The degree of hydrolysis was evaluated by comparison of the N content of the powder before hydrolysis and the N content of the supernatant after hydrolysis and was found to be practically 100 per cent. After hydrolysis the sample was adjusted to pH 2.3 by addition of Na acetate, and diluted to 5 ml. 4 ml. were used for a run.

The sample was added to the top of the column with a bent-tip pipette and washed in with three 0.3 ml. aliquots of buffer pH 2.2. The column had already been mounted on a fraction collector (Model #1205,

RECO Division of the Research Specialties Company, Berkeley, California) and equilibrated at 30° C.

Various trials were carried on to determine the proper way of collecting samples. It was found that the use of "constant volume syphons" was barely satisfactory when 5 ml. aliquots were collected, but lead to errors when collection of smaller aliquots was desired. In our hands, best results were obtained by collecting "time" samples in accurately calibrated test tubes and diluting them to a known volume. The high degree of precision obtained was worth the extra step introduced in the procedure.

The flow of the column was adjusted to about 6.5 ml. per hour, and "fifteen minutes" to "ninety minutes" samples collected with a simple switch of the time-setting apparatus.

After the emergence of serine, the temperature of the water circulating through the jacket was raised to 50° C. At an effluent volume corresponding to twice that of the maximum of the aspartic acid peak, the gradual increase in the pH and ionic strength of the influent was begun. For this purpose, a mixing chamber prepared from a 500 ml. ground joint wash bottle (Corning #1600) containing a 1 1/4 inch plastic-covered stirring bar was mounted over a magnetic stirrer. Such a mixing chamber was interposed between the previously described liter bottle and the column, by means of Tygon tubing.

In assembling the apparatus, the mixing (lower) chamber is first filled to the neck with buffer pH 3.1. The upper part of the ground joint is then inserted, well greased, and secured by rubber bands. Filling of the lower chamber is completed by adding pH 3.1 buffer through the upper

bottle, and applying suction to the top of the upper bottle, to withdraw air from the mixing chamber. When suction is released, buffer flows into the lower chamber. This process is repeated until the air is completely removed from the system. At that moment, the tube connecting upper to lower chamber is clamped and the upper bottle is filled with buffer pH 5.1.

Separation of tryptophan from arginine can be achieved by raising the temperature to 75° after the emergence of histidine. Since our type of hydrolysis is known to destroy the tryptophan, this step was omitted.

Resin was removed from a used column by inversion over a flask connected to a vacuum line. Due to shrinkage which occurred at the 50° C. temperature, columns could not be reused, without prior regeneration with 0.2 N NaOH as previously described.

Since various preparations of resin, as supplied by the manufacturer, had a slightly variable degree of cross-linking, separate runs were made with samples of Dowex 50-X4 and 50-X5, and the relative per cent of each in the "best mixture" calculated by interpolation.

Analysis of the effluent fractions were made by the ninhydrin method described by Rosen (303), which we summarize as follows:

1. Reagents:

   a. Stock NaCN 0.01 M (490 mg/liter).

   b. Acetate buffer. 2700 gm. Na Acetate . 3 H_2O dissolved in about 2 liters H_2O. 500 ml. glacial acetic acid added and the solution diluted to 7500 ml. with H_2O. The pH of this buffer should be 5.3 - 5.4.

   c. Acetate-Cyanide. 0.002 M NaCN in acetate buffer. 20 ml. of sol. 1 diluted to 1 liter with sol. 2.

d. Ninhydrin. 3 per cent in methylcellosolve. This latter should be peroxide-free. Ninhydrin of satisfactory purity was obtained from California Foundation for Biochemical Research. Other preparations we tested were sources of considerable trouble.

e. Diluent. Isopropyl alcohol-water 1:1.

2. Procedure:

One ml. of the sample was mixed with 0.5 ml. solution 3, and 0.5 ml. solution 4. The mixture was quickly heated for exactly 15 minutes in a boiling water bath. Immediately after removal from the bath, 5 ml. of solution 5 were added, care being taken to direct the steam to the center of the test-tube, in order to agitate the solution as thoroughly as possible. Samples were then cooled for exactly 5 minutes in an ice bath, and read at 570 millimicrons (440 for proline) after standing 30' at room temperature.

Using this procedure, since the reaction mixture is strongly buffered, no prior neutralization of the samples is required. The blank or base-line tubes against which the peak tubes were read, was chosen from the tubes immediately before and immediately after the peak itself. In the case of methionine, isoleucine and leucine, the blank must be chosen from the fractions emerging after the peak since in this region, due to the change in pH from 3 to 5, there is a measurable rise in the blank reading. In the vicinity of the ammonia and histidine peaks, traces of ammonia accumulated by the resin from the influent buffers begin to emerge ahead of the ammonia peak, and cause an elevated blank reading. The blank for the determination of ammonia should, therefore, be taken after the NH_3 peak, either before or after lysine. The histidine recovery should be based on a blank reading taken before the emergence of the histidine peak. The NH_3 and histidine determinations by the method used are, therefore, less accurate than the determination of other AAc.

Standard solutions of the various amino acids were made in media comparable to the effluent fractions containing them and were used to evaluate the concentration of each unknown since, even though Beer's Law is followed by all amino acids, their optical density varied somewhat from one particular amino acid to the other.

After determination of the amino acid concentration, samples were pooled by peak for radioactivity measurement. It was found necessary, prior to plating, to desalt the solutions. Omission of this step resulted in a thick salt layer, whose self-absorption was of such a magnitude as to prevent counting. After several trials, we adopted the following desalting technique.

Chromatography columns were prepared from Amberlite IRA 120 (H). The columns were 20 cm. long and contained a reservoir. The pooled samples were concentrated to less than 10 ml. by evaporation at 60° C. and then passed through the columns. Columns were washed with H_2O until the effluent was neutral. At that moment the amino acids were eluted with 300 ml. 1 N NH_4OH. The ammonia fraction was then concentrated to a few ml., and plated on stainless steel cups supplied by Nuclear-Chicago.

Recovery of labelled material after the above type of desalting was tested with a standard solution of C^{14}-Glycine. It was found that the head fraction (H_2O) did not contain any appreciable count. The ammonia fraction contained 5140 counts per minute, corresponding to 98 per cent of the counts driven through the column, while further washing with ammonia eluted only traces of radioactivity.

In Tables 10 to 13 the results obtained using the above technique are presented. Seminal vesicles, temporal muscle, masseter have been

TABLE 10

EFFECT OF CASTRATION AND TESTOSTERONE TREATMENT ON AMINO ACID CONCENTRATIONS AND C^{14} DISTRIBUTION IN GUINEA PIG SEMINAL VESICLES

Amino Acid	Normal		Castrated		Castrated & T.P.	
	µM / 100 mg.	c/m / 100 mg.	µM / 100 mg.	c/m / 100 mg.	µM / 100 mg.	c/m / 100 mg.
Aspartic Acid	52.47	190	30.63	160	50.16	240
Threonine	16.39	200	26.03	200	19.06	198
Serine	39.41	1180	20.18	970	69.13	2020
Proline	-----	----	-----	---	-----	----
Glutamic Acid	74.41	470	54.26	240	71.13	1560
Glycine + Alanine	88.12	6360	81.04	2000	84.48	8740
Valine	39.31	----	39.00	----	32.24	----
Cystine	-----	----	8.70	----	-----	----
Methionine	12.00	----	10.64	----	11.88	----
Isoleucine	25.21	----	18.50	----	21.85	----
Leucine	44.06	----	39.00	----	40.16	----
Phe.Al. + Tyros.	36.91	----	35.20	----	35.04	----
Amide N	46.50	----	42.25	----	40.86	----
Lysine	47.12	----	46.18	----	50.00	----
Histidine	12.50	----	0.00	----	0.00	----
Arginine	40.00	----	26.00	----	53.32	----

TABLE 11

EFFECT OF CASTRATION AND TESTOSTERONE TREATMENT ON AMINO ACID CONCENTRATIONS AND C^{14} DISTRIBUTION IN GUINEA PIG MASSETER

Amino Acid	Normal		Castrated		Castrated & T.P.	
	$\frac{\mu M}{100 \text{ mg.}}$	$\frac{c/m}{100 \text{ mg.}}$	$\frac{\mu M}{100 \text{ mg.}}$	$\frac{c/m}{100 \text{ mg.}}$	$\frac{\mu M}{100 \text{ mg.}}$	$\frac{c/m}{100 \text{ mg.}}$
Aspartic Acid	55.18	190	45.91	110	56.25	180
Threonine	28.48	3	28.38	2	31.12	5
Serine	26.19	530	20.06	310	30.07	600
Proline	15.21	50	14.83	20	14.98	25
Glutamic Acid	69.90	110	74.40	50	93.75	140
Glyc. + Alanine	93.75	1420	73.85	950	87.16	1500
Valine	33.75	____	30.63	____	37.40	____
Cystine	____	____	____	____	____	____
Methionine	10.00	____	6.75	____	6.33	____
Isoleucine	28.50	____	25.12	____	29.84	____
Leucine	56.25	____	50.62	____	56.25	____
Ph.Al. + Tyros.	35.62	____	40.31	____	31.70	____
Amide NH_2	37.50	____	30.52	____	36.52	____
Lysine	58.12	____	56.05	____	53.19	____
Histidine	13.62	____	13.70	____	18.25	____
Arginine	26.94	____	24.75	____	28.48	____

TABLE 12

EFFECT OF CASTRATION AND TESTOSTERONE TREATMENT ON AMINO ACID CONCENTRATION AND C^{14} DISTRIBUTION IN GUINEA PIG TEMPORAL MUSCLE

Amino Acid	Normal		Castrated		Castrated & T.P.	
	µM / 100 mg.	c/m / 100 mg.	µM / 100 mg.	c/m / 100 mg.	µM / 100 mg.	c/m / 100 mg.
Aspartic Acid	58.06	27	42.84	17	56.30	17
Threonine	31.87	12	28.16	14	30.40	13
Serine	27.15	198	25.00	92	29.23	140
Proline	18.12	9	13.12	7	18.72	15
Glutamic Acid	66.31	42	61.62	42	69.24	46
Glyc. + Alanine	83.43	702	59.00	738	71.00	900
Valine	36.53	---	31.24	---	43.20	---
Cystine	-----	---	-----	---	-----	---
Methionine	13.20	---	14.70	---	12.70	---
Isoleucine	24.65	---	16.51	---	23.89	---
Leucine	41.68	---	35.85	---	45.29	---
Phe.Al. + Tyros.	34.46	---	34.56	---	37.00	---
Amide NH_2	40.81	---	32.48	---	38.98	---
Lysine	22.12	---	29.36	---	24.99	---
Histidine	12.84	---	10.46	---	11.66	---
Arginine	27.72	---	27.10	---	29.98	---

TABLE 13

EFFECT OF CASTRATION AND TESTOSTERONE TREATMENT ON AMINO ACID CONCENTRATIONS AND C^{14} DISTRIBUTION IN GUINEA PIG RECTUS FEMORIS

Amino Acid	Normal		Castrated		Castrated & T.P.	
	µM / 100 mg.	c/m / 100 mg.	µM / 100 mg.	c/m / 100 mg.	µM / 100 mg.	c/m / 100 mg.
Aspartic Acid	54.87	60	56.85	40	54.82	50
Threonine	30.36	120	30.00	80	32.80	120
Serine	27.52	400	26.35	390	30.28	430
Proline	14.16	20	13.81	20	13.25	70
Glutamic Acid	87.51	100	78.92	120	85.98	130
Glyc. + Alanine	85.72	1530	88.15	1480	95.28	1500
Valine	35.81	----	35.59	----	34.19	----
Cystine	----	----	-----	----	-----	----
Methionine	12.80	----	13.62	----	13.34	----
Isoleucine	29.30	----	29.10	----	27.34	----
Leucine	50.45	----	57.60	----	54.75	----
Phe.Al. + Tyros.	31.85	----	33.60	----	36.65	----
Amide NH_2	39.39	----	28.35	----	47.80	----
Lysine	60.12	----	69.35	----	57.10	----
Histidine	12.15	----	14.00	----	11.99	----
Arginine	25.15	----	22.59	----	23.32	----

chosen as samples of organs responsive to testosterone stimulation. Unfortunately, no more protein powder was available for chromatographic analysis of prostate and retractor penis, both of which are very responsive. As an example of unresponsive organ, rectus femoris has been chosen.

From the analysis of the data presented in Tables 10 - 13, we see that a satisfactory separation of all but two pairs of amino acids is achieved. The glycine-alanine and the phenylalanine-tyrosine pairs are separable, as long as quantities of powder are used not larger than 3 mg. This quantity would have been too low for a satisfactory counting, and we were forced to a compromise.

Tables 10 - 13 indicate that the amino acid composition of seminal vesicles is substantially different from that of muscle, the former being poorer in threonine and richer in lysine, to mention only the most evident findings. It appears thus convenient, also in the light of the somewhat different biological behavior, to examine seminal vesicles separately from muscles.

It can be seen from Table 8 that radioactivity was found only in the glycine, serine, glutamic and to a less degree, in aspartic and threonine. These amino acids are linked by known metabolic pathways leading to the interconversion of each one into the others. This same distribution is seen in all the muscles tested as well.

Both concentration and count of aspartic acid are decreased by castration and increased by testosterone treatment. This behavior is followed by serine and glutamic acid. Isoleucine and arginine concentrations are appreciably decreased in Group II and return toward the

normal in Group III. Histidine was found only in Group I, while cystine appeared only in Group II. Threonine concentration, but not count, appeared to be increased in Group II. Glycine-alanine concentration were not modified by either castration or androgen administration, but the relative count was decreased to less than 30 per cent of the normal value in Group II and increased to 130 per cent in Group III. The concentrations of the remaining amino acids did not appear modified by either treatment.

Composition of the various muscles obtained from normal animals appears relatively constant.

Aspartic acid concentration and count were decreased in both the "responsive muscles" (masseter and temporal) but not in the "unresponsive." This same pattern was more evident in serine, glutamic and glycine-alanine. Serine behaved somewhat differently from the other amino acids of this group insofar as the variations were mostly in the count, reduced to about 50 per cent in Group II, while the concentration was not modified to a comparable degree. Here again, we have no response, in either counts or concentration, in rectus femoris.

The remaining amino acids did not respond, or presented contradictory behavior.

CHAPTER III

CONCLUSIONS

We have studied the effects induced by castration and by testosterone propionate administration on several parameters related to protein synthesis. Our experimental animal has been the guinea pig, for the reasons illustrated in Chapter I. Before entering further into the discussion of our results, we wish to recall several aspects of our experiments to make the evaluation of their validity and limitations more immediate.

Fifty-seven different organs have been dissected from each guinea pig and studied. This type of approach afforded a rather complete search for possible effects, which needed not be the same in different sections of the body - as our results have indicated - but limited the number of animals that could be studied. This way, only major changes could be located. We were, in other words, scanning the field with a low power magnification, which permits rapid evaluation of all major accidents. Some of the things we have seen are, we think, probably truly existent. For some others, evidence was only suggestive, and a higher magnification will be needed.

The first parameter studied has been the weight. It has already been studied extensively, and offered to us the advantage of a road already

mapped in part. Summarizing again what has been shown in the guinea pig, but not necessarily in other species (282), there are organs "responsive" to the injection of testosterone and organs which, after administration of the androgen, do not show any appreciable variation of their characteristics. Several of the "responsive organs" such as seminal vesicles, prostate and retractor penis have a well known sexual function. For other, like the masticatory muscles, no sex function is immediately evident. Such a function has been claimed for these muscles by some authors. This question, though interesting per se is immaterial and extraneous to our problem.

One question which arises immediately is the following: What does the growth of the responsive organs mean, at a cellular and subcellular level? Is the growth due to increased number of cells or to an increased cell volume? Has testosterone an effect on the cell division mechanism or is it effective only on the size of cells, on the amount of protoplasm that each cell contains?

Some information along these lines is provided by yet unpublished observations by Kochakian (293), who has seen that castration results in a decreased size of muscular fibers. We have contributed some data on the fiber composition and dynamism, as we shall discuss below.

The injection of labelled precursors offered us the opportunity of studying the effect of the hormone on the all important step represented by precursor uptake. In preliminary trials we have tested C^{14}-aspartic acid and 2-C^{14}-glycine, and found that glycine was more satisfactory for our purpose.

We have found that labelled glycine is taken up by every organ

tested, thus indicating an underlying basic mechanism independent from the presence of testosterone. This is not surprising if we recall that castrated animals are still capable of reaching a size which represents a large fraction of the size of the normal animal, thus indicating protein synthesis independent from androgenic stimulation.

We have also found that the growth induced by testosterone in the responsive organs is coexistent with an increased uptake, thus revealing the presence in these organs of a subsidiary mechanism dependent on the presence of testosterone. Although the hypothesis that testosterone is responsible for the synthesis of a specific protein present to an appreciable extent only in the responsive organs would seem very appealing, any conclusion in this sense is so far premature.

Fractionation of some of the responsive organs has shown interesting points. The decrease of aspartic acid concentration and labelling seems to be a general consequence of castration in all the responsive organs tested. Thus, we have a reduction in seminal vesicles, a reduction in temporal muscle, a reduction in masseter, while no modification is seen in the unresponsive rectus femoris. This effect is offset by the administration of testosterone.

Very interesting appears the behavior of the glycine-alanine peak. It was found in seminal vesicles that, while no apparent variation in the concentration of the two amino acids could be demonstrated after castration, there was a very significant reduction in the C^{14} incorporation, thus indicating that the "rate of exchange" was primarily effected. This was not true in the case of the responsive muscles, where a decrease in both concentration and count was seen.

The transfer of carbon from glycine to serine was also influenced by the removal or addition of testosterone. Thus, in seminal vesicles, while castration resulted in a decrease of 50 per cent in the serine concentration, and of about 20 per cent in the count, the administration of testosterone produced an increase of about 90 per cent with respect to the control values in the concentration and a proportional increase in the count. This effect is even more striking if the increments are computed as per cent of the value observed in the castrated animal. In this case, we would have a 300 per cent increase in the concentration and a 230 per cent increase in the count. A similar pattern is seen in the two responsive muscles. In this latter case, though, the count was affected slightly more than the concentration.

It must be recalled at this time that the glycine peak, source of the C^{14} for the serine, behaved differently in muscle and seminal vesicles, insofar as only count depression or stimulation was seen as a consequence of castration and testosterone administration in the latter, while in the former both concentration and count were depressed. This means that, in the case of seminal vesicles while the decreased concentration of serine in the presence of a non-modified concentration of its precursor in the castrated animal, would definitely indicate a block in the interconversion, it is difficult to assess to which extent the decreased serine count is due to a block and to which extent it is due to a decreased specific activity of the precursor.

In the case of muscle, the existence of a block to the glycine-serine interconversion is more difficult to assess, due to a decreased concentration in the glycine-alanine peak. Before any conclusion could

be drawn, it is necessary to separate glycine from alanine and identify the amino acid(s) responsible for the decreased concentration. With these premises, it is entirely possible that a situation similar to the one existing in the case of seminal vesicles would exist also in the case of muscle.

Another amino acid modified by testosterone levels is glutamic acid, whose counts and concentration decrease in seminal vesicles as a result of castration, to return to the normal values (concentration) and above the normal values (count) after injection of testosterone. This same effect was seen in masseter as well, while in the temporal muscle, it was not evident.

It is interesting that glycine, serine, aspartic acid and glutamic acid, all of which are modified by testosterone removal and restoration, are the only ones in which radioactivity was found and are also related by well known interconversion reactions.

This would indicate that the effects seen are possibly not the only effects elicited by testosterone. Others could exist concerning the transfer of label within different "families" of amino acids. For instance, it would be of interest to explore the significance of the disappearance of histidine in seminal vesicles after castration. Also, the isoleucine-leucine system appears worth exploring.

It must be remembered that, in our experiments, only the mixed proteins were analyzed. Most of our results could be interpreted in terms of disappearance or of synthesis of particular proteins, especially rich in one or the other amino acid. This question must be settled before sound conclusions could be reached. Our experiments have indicated

several possible loci of action for testosterone. Further experimentation is needed, but it is not unlikely that the point of attack of testosterone will finally be identified in one of those which we have indicated.

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